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APPARATUS FOR THE STUDY OF REDOX POTENTIAL IN BIOLOGICAL SYSTEMS*

By J. PERCY BAUMBERGER

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(Accepted for publication, May 25, 1933)

The four pieces of apparatus[1] described in this paper have been used in several investigations on biological oxidation-reduction systems. Other apparatus for similar purposes have been described by Ahlgren (5), Lehmann (4), Clark (1), Michaelis (2), Borsook and Schott (3), and Baumberger, Jürgensen, and Bardwell (7).

1. The Modified Thunberg Tube.—Ahlgren (5) has described in detail the use of the Thunberg tube in the study of the reduction of dyes by biological systems. The apparatus shown in Fig. 1 has the advantage that it is cheaper, has no ground glass stopper to be greased and thus involves no special difficulty in cleaning, and will hold a vacuum suitable for the development of extremely low redox potentials such as the complete reduction of rosinduline. The experimental procedure with this tube is to introduce the substrate, buffer, and dye and finally the enzyme, then to apply de Khotinsky cement to the pyrex vacuum tube at point *DeK*, warm the tip of the soft glass adapter *b*, and introduce it into the cement so that it rises in the interstices between *b* and the neck. Cool the cement, attach *b* to water pump, and evacuate to boiling for a minute. It is essential to keep the tube warmer than the tap water and to wet the walls with the contents, as thereby the oxygen is more readily displaced. Heat *b* at the constriction while still evacuating and finally seal off the attached lower half of *b* and anneal with care. (The introduction

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* This work was supported in part by a grant from the Rockefeller Fluid Research Fund of the Medical School of Stanford University and by a grant from the De Lamar Fund of Harvard Medical School.

[1] This apparatus was skillfully made by Macalaster-Bicknell Co., Cambridge, Massachusetts.

of glass capillaries through *b* should be avoided, as scratching of the inner surface invariably follows and leads to cracking while heating.) After observing reduction time, or final color if a multiple dye system has been used, the de Khotinsky cement may be heated, the tip pulled off, and the tube washed. The remaining cement can be repeatedly used or removed with alcohol.

2. *The Modified Borsook and Schott Tube.*—Borsook and Schott (3) describe a vacuum electrode tube which can rock in a vertical position in such a way as to be in contact with a reference half-cell through a capillary tube (*a*, Fig. 2) filled with KCl agar-agar which will stand an atmosphere of pressure. A battery of such tubes may be made to rock with the salt bridge capillaries dipping in a large dish of KCl to which a calomel half-cell is connected. The changing oxidation-reduction potential of a system consisting of substrate, enzyme, and dye may be followed conveniently by measuring the P.D. between the two half-cells. When the P.D. no longer changes with time, equilibrium has been reached and the redox potential observed is a function of the free energy of the system. Borsook and Schott's tube has been modified by substituting the evacuation sealing system for ground glass stoppers as in (1) by adding a boot, *b*, at the tip so that satisfactory circulation of the contents may be obtained, which is particularly advantageous in work with slices of tissue; and by introducing the platinum electrode, *Pt*, into the boot instead of having it borne by the glass stopper where it would be exposed to breakage and contamination.

3. *Reduction Burette.*—The usual apparatus (Clark (1)) for the reduction and transfer of reduced dyes is very intricate, expensive, fragile, and immobile. The reduction burette here described is much less so in each of the four respects mentioned and is adequate for its purpose. It (Fig. 3) consists of a 25 cc. burette surmounted by a 200 cc. bulb from which it is separated by a perforated glass plate, *P*. A special stop-cock, *S*, at the top of the burette and a ground glass stopper at the top of the bulb complete the reduction burette. In operation the dye solution and wet[2] platinized asbestos are introduced into the bulb, the ground-in stopper is fastened in with a rubber band, the

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[2] There is danger of explosion when dry platinized asbestos is introduced from air into a hydrogen atmosphere.

burette inverted, and the dissolved oxygen removed by water vacuum pump through side stop-cock *S*, which at the same time is connected

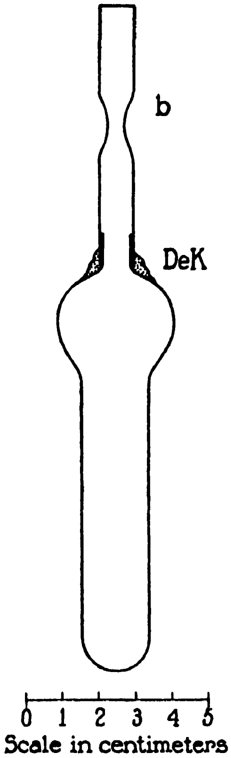

Fig. 1

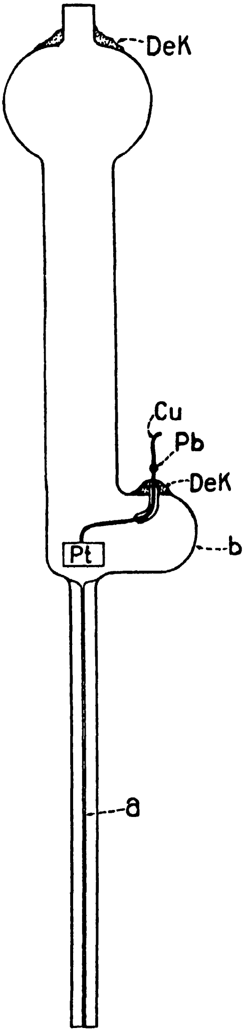

Fig. 2

Fig. 1. In all the text-figures the scale is the same as that given in this figure.

by means of a *Y* tube to a Kendall (6) furnace through which oxygen-free hydrogen and nitrogen can be obtained. When the solution has boiled, the burette is allowed to fill with hydrogen, the stop-cock is

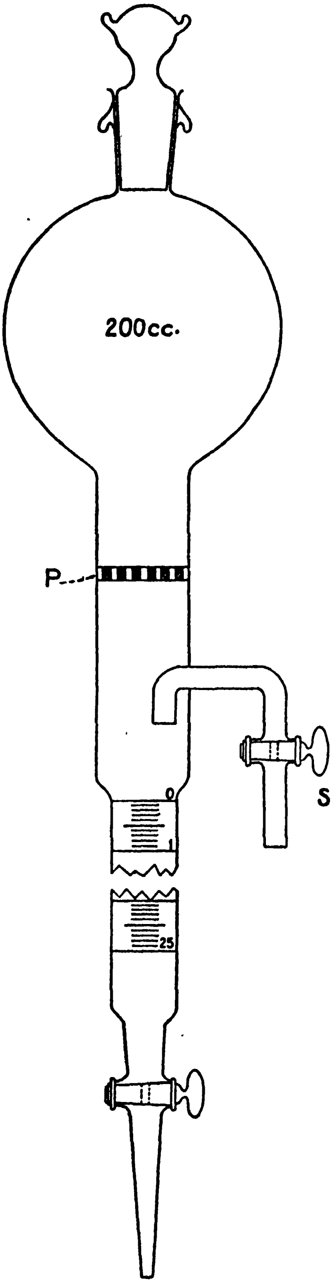

FIG. 3

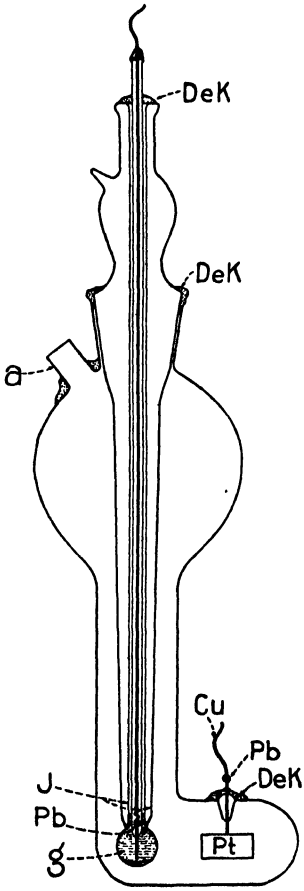

FIG. 4

closed, and the burette is detached from the vacuum pump. With the burette still inverted, the fluid is gently rotated to bring it into contact with the suspended platinized asbestos. When the dye is fully reduced, the reduction burette is again attached to the *Y* tube through *S*, the hydrogen is removed by evacuation, the burette is filled with pure nitrogen and evacuated a second time, and then finally filled with nitrogen. The next step is to slowly turn the burette right-side up and to allow the asbestos to settle on the glass filter. Create a vacuum up to the side stop-cock, open this stop-cock for an instant so that the gas pressure in the burette will become less than in the bulb, and the fluid will be driven down into the burette. When the burette is filled and the bulb dry, raise the nitrogen pressure to about 1.20 atmospheres so that the superpressure will expel the contents of the burette as needed.

The same form of burette is convenient for handling oxidized dyes from which dissolved oxygen may be removed by vacuum and the dye put under nitrogen pressure.

4. Vacuum Oxidation-Reduction Cell.—The cell shown in Fig. 4 is suitable for the study of the oxidation-reduction processes described under (2) above, but it has special features which adapt it particularly to the study of these processes in cell suspensions and in the fluid surrounding slices of tissue. This cell has the features of vacuum seal, boot, and platinum electrode mentioned in (1) and (2), and in addition contains a glass electrode as reference half-cell. The cell has the advantage that it does away with the salt bridge and thus removes the contact potential error resulting from long contact of a salt bridge with a physiological solution. Progressive pH changes, resulting from cell metabolism, are cancelled out in those systems in which the redox potential is affected by pH in the same degree as the hydrogen electrode. Any changes in ionic strength or in temperature are effective on both half-cells and thus such changes tend to cancel out rather than to augment potential differences. The potential difference due to changes in the redox potential on the platinum electrode is thus made the chief variable. The actual pH of the solution may be determined before and after the experiment by connecting the solution to a calomel half-cell by a salt bridge introduced through *a*, and measuring the P.D. between the glass electrode and the calomel half-cell. If the particular glass electrode gives a P.D. of 0.100 in a buffer of pH 3.97 at 30°C. and a P.D. of 0.160 in the unknown, the pH of the unknown will be $0.160-0.100/0.060 + 3.97 = 4.97$. Perfect glass electrodes behave in this manner, within certain pH limits (Dole (9)), if no "deviation film" effects are present (Kahler and DeEds (8)). "Deviation film" effects are avoided by supporting the thin bulb of Corning 0.015 glass by a double walled tube of soft glass blown so as to fit a ground glass joint at the top. The air jacket (*J*) thus formed limits the effective contact of the outside solution to the bulb itself. The glass electrode bulb *g* contains KH phthalate buffer to which quinhydrone has been added in excess; a platinum electrode sealed into a glass tube projects into this solution.

The P.D.'s between the glass electrode and the platinum electrode are measured by means of a thermionic electrometer designed by R. K. Skow in my Laboratory. Any form of electrometer suitable for determinations with the glass electrode may be used with the vacuum oxidation-reduction cell.

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CRITICAL FREQUENCY OF FLICKER AS A FUNCTION OF INTENSITY OF ILLUMINATION FOR THE EYE OF THE BEE

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I

The use of the reaction of the honey bee to a moving stripe system for testing visual acuity and intensity discrimination has been described in detail in previous papers (Hecht and Wolf, 1928–29; Wolf, 1932–33 *a*, *b*; Wolf and Crozier, 1932–33). The motion of the striped pattern under the experimental conditions was always such that a sudden but not too rapid lateral displacement was made, to which the bee reacted by moving from its straight course of progression with a sharp turn against the direction in which the pattern was moved. For different visual acuities, threshold intensities were determined for this response, and in the case of intensity discrimination the minimal difference in brightness of alternating stripes was found at which the bee reacts by the characteristic reflex.

In previous experimental tests the speed of translocation of the pattern in front of the bee's eye was kept so small that no fusion of the alternate stimuli could take place. Experiments concerning the flicker phenomenon in the facetted eye of an arthropod have thus far been made only with larvae of the dragon-fly (*Aeschna cyanea*; Sälzle, 1932). As index of this animal's reaction to flicker there was used a reflex, the throwing forward of the labium toward a moving object, which occurs as long as the speed of repeated flashes of light is below the critical frequency for fusion of the sensory effects. Having thus only one source of information about the number of single impressions which can be perceived separately by an insect's eye, as a function of illumination, the critical fusion frequency of intermittent stimulation by light was studied in the honey bee. Using the bee's reaction to moving patterns we have a means of more direct approach for test as compared with the "catch" reaction in the dragon-fly, and a certain body of precise information is already available concerning the relationship between visual acuity and illumination for this eye.

Bees are positively phototropic and negatively geotropic; they tend to creep upward upon a transparent inclined surface which is illuminated from underneath. If a visible pattern be moved below the creeping plane, we obtain a typical response to the displacement of the pattern, as described. The bee's response to a moving stripe system, however, can be obtained if the system is not shifted while the bee begins its journey up the inclined surface, and even while the pattern is in continuous motion in one direction of constant speed. The bee's path then involves a continuous creeping against the moving stripes, from the lower edge of the field to one of the sides of the compartment, at a fairly low angle of slope. In case the bee enters the field from the side against which the stripes are moving, it runs straight across the field to the other side of the compartment without showing any tendency to creep upward; if entering from the opposite side, *i.e.* moving in the direction of the moving pattern, it takes a sharp turn of almost 180° and crawls quickly out of the field, up the walls of the chamber, continuing to walk hanging from the cover of the compartment until it again enters the field from below or from the other side, then showing the same reaction once more. At very high speeds of motion of the pattern, however, it quite often happens that the bee, apparently unable to move against the stripes, "swims" so to speak with the stream with excessively rapid movements of the legs. In spite of a certain variation in the behavior of different individuals, the critical intensity of light and frequency of flicker at which the bee shows the first definite reaction to the moving pattern under varying experimental conditions can be determined rather accurately.

This reaction of the bee was employed in the following way. For pattern plate a round mud-ground glass plate, 50 cm. in diameter, was used. On the ground surface, which is the upper side of the disc, 20 sectors of opaque black paper are glued, in such a way that there is made a sector wheel with 20 black and 20 translucent sectors of the same size. The breadth of each sector on the central side of the disc is so chosen that the visual angle sustained by it is great enough so that the sectors passing underneath the creeping plane can be reacted to at the lowest intensities used during the test (Fig. 1). With a sector wheel, for which the angular speed is the same at any distance along the radius, the flicker frequency for the bee is the same regardless of its position nearer to the center of the disc or to the periphery. The peripheral part of the ground glass disc which is in the field of vision of the bee is illuminated by light reflected from a mirror underneath, which gives an even illumination of the bee's visual field (Fig. 1). The source of light is a 1000 watt concentrated filament lamp which can be placed in three different positions on an optical bench. The positions are 15 cm., 55 cm., and 175 cm. from a diffusing screen in the wall of the dark room containing the apparatus. The amount of light admitted into the dark room is controlled by an accurately calibrated diaphragm for each position of the source. The intensities available on the upper surface of the ground glass wheel are measured

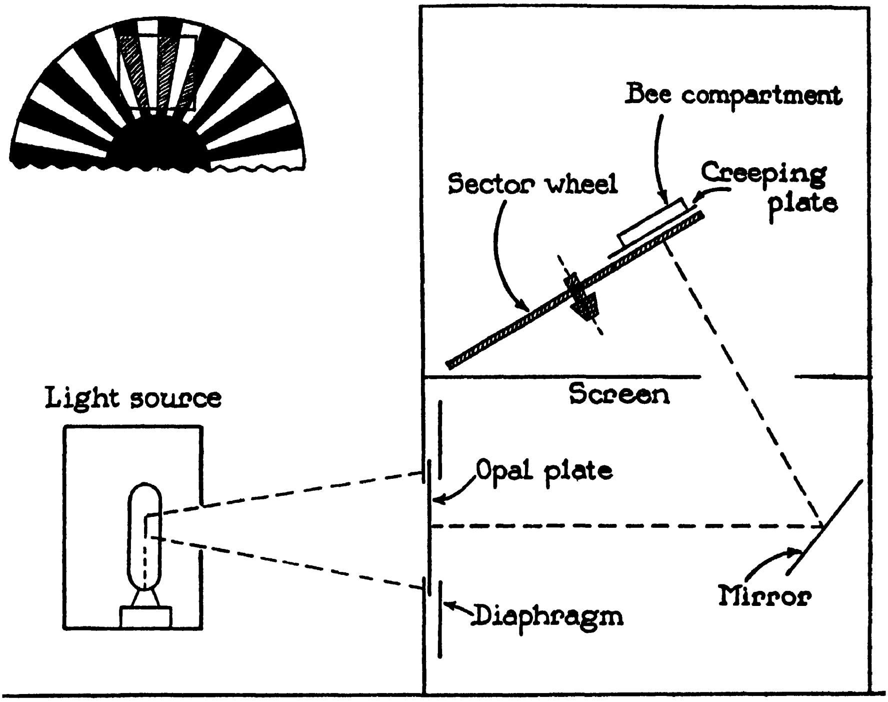

FIG. 1. Diagram of apparatus for measuring critical frequencies of flicker at different light intensities.

by means of a Macbeth illuminometer. The intensity values obtained for the different settings of the diaphragm and positions of the light source are plotted against the scale readings of the diaphragm. Thus three smooth calibration curves are obtained. From these there could be read with sufficient accuracy the threshold intensities at which the bees just give the first noticeable response to the motion of the stripes at given velocities of rotation of the disc.

The ground glass plate is mounted on an axle running in ball bearings and rotated smoothly by a D. C. motor of which the speed is controlled by a rheostat and transmitted by a system of reduction gears to the disc. By using pulleys of different diameters for transmission, and by adjustment of the rheostat, the velocity of rotation could be so varied that almost any speed was obtained, providing flicker frequencies between 2 and 70 per second. This range proved to be wide enough for study of the critical frequency of flicker over a range of intensities of 4 logarithmic units.

During test a bee, after the wings have been clipped, is put into the compartment, above the rotating disc. The speed of rotation is adjusted for a certain value, measured with a stop-watch, and kept constant. The variation in speed is negligible, as repeated stop-watch readings before, during, and after the test showed. The bee then creeps over the illuminated field and reacts in the typical manner to the sectors passing by, provided the light intensity is high enough. By opening or closing the diaphragm the intensity is found at which the bee just begins to show a reaction to the motion of the visual field. For this threshold response at a given flicker frequency the associated threshold intensity is measured. This test is repeated 10 times for each given flicker frequency, with 10 different bees, to give a mean value represented as one point on the curve illustrating the relation between flicker frequency and illumination (Fig. 2). It has been shown previously that on account of the uniformity of the members of a colony of bees it is justifiable to take bees for single tests only once, instead of repeatedly (Wolf, 1932–33 *a*, *b*).

II

Flicker frequencies were chosen from 2.4 to 52.6 flickers per second, 18 selected frequencies covering this range. Some tests were made at higher frequencies, up to 68 per second, but responses of the bees could not be obtained. At a flicker frequency of 55.3 per second 3 bees gave uncertain reactions at the highest intensity of light available. Many other bees which were tested did not react at all. It seems therefore fairly certain that the minimum interval for intermittent stimulation, such that the stimuli can be reacted to separately, is at a frequency of about 55 per second. This value compares nicely with the findings for the dragon-fly (Sälzle, 1932); the maximum value found there was 59.7 per second.

TABLE I

Critical Intensity of Illumination for Threshold Response in the Bee as Function of Frequency of Flicker

Frequency of flicker per sec.	I	P.E.$_I$
	millilamberts	
2.4	0.011	±0.0018
3.1	0.019	0.0019
4.0	0.033	0.0018
6.0	0.064	0.0040
8.0	0.111	0.0056
10.6	0.171	0.0043
13.3	0.256	0.0161
17.2	0.546	0.0419
21.7	0.706	0.0306
23.8	0.761	0.0902
27.0	0.915	0.0634
31.7	1.270	0.0699
35.7	1.281	0.0837
38.4	1.633	0.0600
42.5	2.956	0.0633
47.6	7.474	0.587
50.0	11.85	0.711
52.6	57.66	3.682

The data for critical flicker frequency at different intensities between 1/100 and 100 millilamberts are presented in Table I. The values for threshold intensities are *mean* values for the number of bees tested in each case (n = 10), with the probable errors of the threshold intensities.

The data show that at low flicker frequency the intensity for the threshold response is small. As the flicker frequency is increased, the intensity has to be increased, at first more rapidly and then only slightly, with relatively great increase of frequency, until finally at very high flicker frequencies the increase in intensity is again greater.

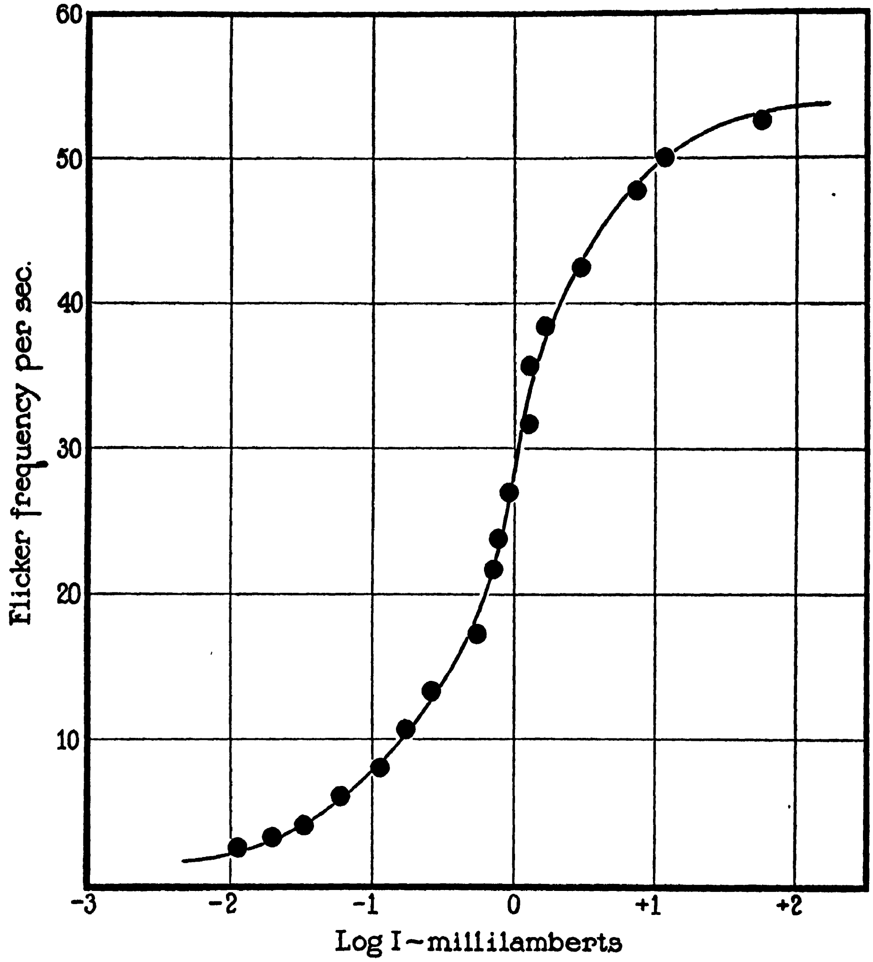

FIG. 2. Relation between critical flicker frequency and illumination. Single readings from 180 bees at 18 different flicker frequencies. The points represent averages of 10 tests.

The data are plotted in Fig. 2, where the flicker frequency is plotted against the logarithm of intensity. Fig. 2 shows that the points representing the mean values fall on a smooth S-shaped curve. This curve has great similarity to the ones given by Sälzle (1932) for the dragon-fly, and to the visual acuity curve for the bee (Hecht and Wolf, 1928–29; Wolf, 1932–33 *b*).

In comparing the flicker curve for the bee with the visual acuity curve it is significant that the threshold for visibility in the visual acuity tests and the lowest intensity at which the bee reacts to a very low flicker frequency are identical. By using still lower frequencies for flicker, and decreasing the intensity correspondingly, no reaction of the bee is obtained. The bee starts to react as soon as the intensity is above 0.007 millilamberts with flash frequency above 2.4 per second. At a speed of less than 2.4 sectors per second passing in front of the bee's eye, no reaction could be obtained even at higher intensities. While the bee is moving freely in the compartment the slow motion of the sectors is negligible compared with the bee's own velocity of progression, so that no reaction takes place. We thus have to assume that at least two changes from light to dark have to take place to cause the reaction of a bee which is permitted to move freely.

With higher flicker frequencies the threshold intensity for response increases. In comparing the flicker curve with the visual acuity curve it can be shown that the increase is in the two cases identical. The inflection point of the flicker curve occurs at an intensity of about 1 millilambert, which is found also for the visual acuity curve (Hecht and Wolf, 1928–29; Wolf, 1932–33 *b*). At higher flicker frequencies, above the inflection point, the intensity has to be increased more rapidly per unit increase in flicker frequency to cause the bee to react, until at a frequency of about 54 flickers per second the curve reaches a maximum level and flattens out, the intensity for the response being very high. This intensity corresponds fairly well to the highest intensity for visual acuity tests at which the bee's eye reaches the maximal resolving power.

For further comparison of the flicker curve with the visual acuity curve the following considerations are of importance. It has been assumed for the human eye (Hecht, 1927–28) and the bee's eye (Hecht and Wolf, 1928–29) that the mosaic of retinal elements, or in case of the facetted eye the ommatidia, have different thresholds. The increase in resolving power of an eye at higher intensities can thus be explained by the assumption that with increasing intensity more and more elements are actively functioning. The elements with different thresholds being distributed over the eye at random, with higher intensities it is to be assumed that elements with the same threshold lie closer together and provide thereby a higher visual acuity.

In studying the relations between threshold intensities and critical flicker frequencies the same threshold relations for the different ommatidia must play a part during response. At low intensities, while only few elements are functional and thus very much wider apart from each other than at higher intensities when others come into play, bees can react only to a slow flicker frequency, giving the reacting elements far apart time enough to be stimulated over a certain period to cause a photochemical effect during stimulation and to come back to the original threshold condition during the period of darkness. If at a low intensity the flicker frequency is made greater, the time of exposure to light for the elements with a low threshold and the corresponding period in dark would be smaller than necessary for causing adequate photochemical effect in the retinal element or for providing enough time to build up in darkness enough new photosensitive material necessary for reaction to the next flash of light. This means that the elements come to a stationary condition where light and dark reactions come to an equilibrium without setting off any impulses during illumination (Hecht, 1922–23, 1931; Hecht and Wolf, 1931–32; Sälzle, 1932).

As the intensity is gradually increased the bee is able to react to higher and higher flicker frequencies. With higher intensities the thresholds of more and more elements of the ommatidial mosaic are reached. As their distribution at random calls for the assumption that the distance between functional elements becomes smaller and smaller, faster transitions from the unexciting to the exciting state of the ommatidial surface can be reacted to. At an illumination such that the thresholds of all elements are exceeded, the maximum capacity of perceiving flicker singly is arrived at. As with increasing intensity more elements with higher thresholds come into play, the amount of light impinging upon such elements is greater, causing a photochemical effect during which the photosensitive material present is decomposed. During the period in darkness, which with higher flicker frequency is shorter, the photosensitive material is replenished probably by a reaction of second order following the mass law. Thus for elements of higher and higher threshold the period in the dark needs to be shorter to bring them back to their original condition of excitability. Consequently the bee can react to higher frequencies of flicker at higher intensities. If, however, the flicker frequency is increased still further, which gives each element again shorter times of exposure and shorter periods for recovery, we again find the elements in a stationary state during which light and dark reactions come to an equilibrium not affected by any one flash of light because the photosensitive material is below threshold concentration, nor replenishing during the dark period any considerable amount of photosensitive material, because the light reaction does not really get started, and consequently does not cause any reaction in the opposite sense. Under these conditions the flickering visual field for the bee has the effect of a stationary one uniformly illuminated, and no reaction to the rapidly moving sectors is obtained.

III

The variability of the determination of the threshold intensities at which the bee begins to react to different flicker frequencies is of particular interest. In previous tests on intensity discrimination of the bee in relation to visual acuity (Wolf, 1932–33 *a*, *b*; Wolf and Crozier, 1932–33) it was shown that the *variation* of the increase in light intensity is a function of the width of the stripes and of the illumination. The analysis of the data indicated that the amount of variation depends in part upon the frequency of alternate stimulation of the ommatidia of the bee's eye.

In testing the bee's reaction to different flicker frequencies at different illuminations, a study of the variation of threshold intensity necessary to give threshold response gives further support to the assumption that the variation depends on the frequency of transition of the retinal elements from one state of excitation to the other.

For the threshold intensities which just elicit the first response of the bee, the probable errors were computed according to Peter's formula (Table I). With increasing flicker frequency from 2.4 to 52.5 per second, the probable error increases over a thousand times.

The logarithm of P.E.$_I$ is plotted against flicker frequency in Fig. 3. The points for P.E.$_I$ at different flicker frequencies fall on an inverse S-shaped curve, compared with the flicker curve given in Fig. 2. Log P.E.$_I$ for threshold intensity increases smoothly for flicker frequencies between 2.4 and 25 per second. For frequencies between 25 and 42 per second P.E.$_I$ keeps more or less on an even level and rises more rapidly than for the lower part of the curve for frequencies above 45. Comparing the respective points for different flicker frequencies, in Figs. 2 and 3, it is apparent that in the lower part of the curve in Fig. 2 for a relatively small increase in flicker frequency the increase in intensity has to be rather great for causing threshold response; in Fig. 3 over the same range of flicker frequencies P.E.$_I$ increases smoothly. The middle part of the flicker curve (Fig. 2) is steep, which means that over a relatively wide range of increasing flicker frequency the increase in intensity needs to be only slight. For the corresponding flicker frequencies, in Fig. 3, log P.E.$_I$ increases only very little which corresponds to the small increase in intensity over the steep

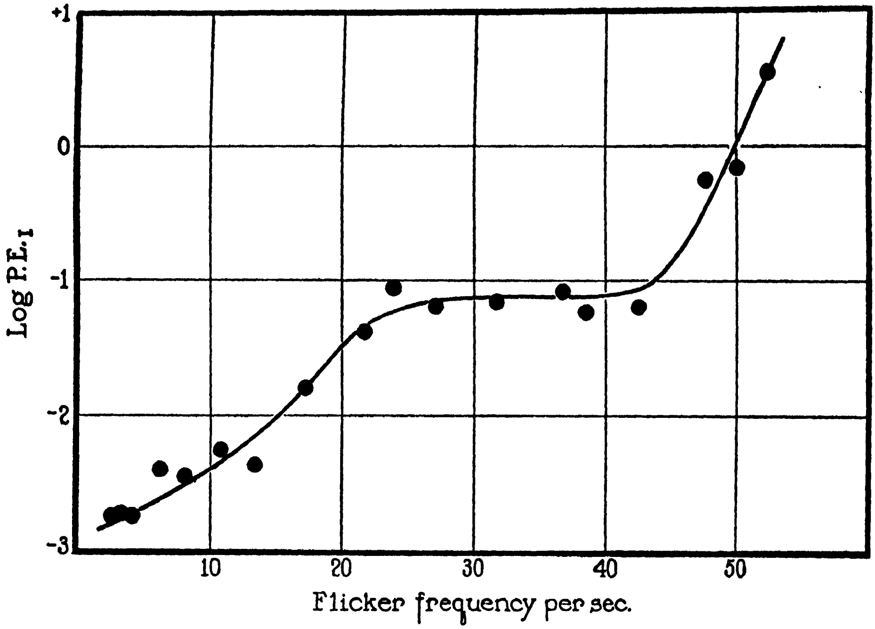

FIG. 3. Relation between the logarithm of the probable error of threshold intensity I and flicker frequency.

part in Fig. 2. The flicker curve above the inflection point tends to flatten out rather rapidly, with increasing flicker frequency, which means in terms of intensity that its increase has to be rather great for a small increase in flicker frequency to cause the bee to react. The corresponding part for flicker frequencies in Fig. 3 shows that P.E.$_I$ increases steeply up to the highest frequency at which the bee was found to give a definite reaction.

This variation of threshold intensity in relation to flicker frequency can be interpreted with the help of our previous findings (Wolf, 1932–33 *a*; Wolf and Crozier, 1932–33). At low intensities and the corresponding low flicker frequencies, where only few elements are concerned—for the others the intensity is below threshold—the occurrence of alternate stimulation of the functional elements is small. As the intensity increases and more elements come into play the critical flicker frequency increases and with it the occurrence of alternate stimulation of elements, for which reason we have to expect an increase in the variation of the threshold intensity (*cf.* Wolf and Crozier, 1932–33), up to the point where for relatively small increase in flicker frequency the intensity increase had to be great. For the range over which increase in flicker frequency is high but increase in intensity small—which means that only for few additional elements the threshold is reached and consequently the increase in alternate stimulation is only slight—P.E.$_I$ stays almost at an even level. For any further increase in flicker frequency the intensity has to be raised more rapidly again. New series of elements come into function by which the frequency for alternated stimulation for neighbor elements is growing and with it the probable error for measured intensity for threshold response. This agrees in a very striking way with the analysis previously given (Wolf and Crozier, 1932–33) upon the basis that the variation of the measured intensity for threshold response depends upon the intensity of the excitation induced, and that in this intensity there are two distinct elements; namely, the intensity of illumination and the frequency of exposure.

In Fig. 4 the data on variation are presented in still another way. The graph shows that the P.E. for threshold intensity is a power function of the intensity (*cf.* Wolf, 1932–33 *a*). This relationship will be understood readily by remembering that any increase in illumination (ΔI) calls for addition of elements to the set of functioning ones already concerned at that particular intensity. As more elements are involved, the chance of alternate stimulation of neighbor elements increases, and with that $P.E._I$ has to increase according to our previous considerations. Fig. 4 thus gives an illustration of the dependence of the variation of the threshold intensity upon the flicker frequency from a different point of view than is concerned in Fig. 3.

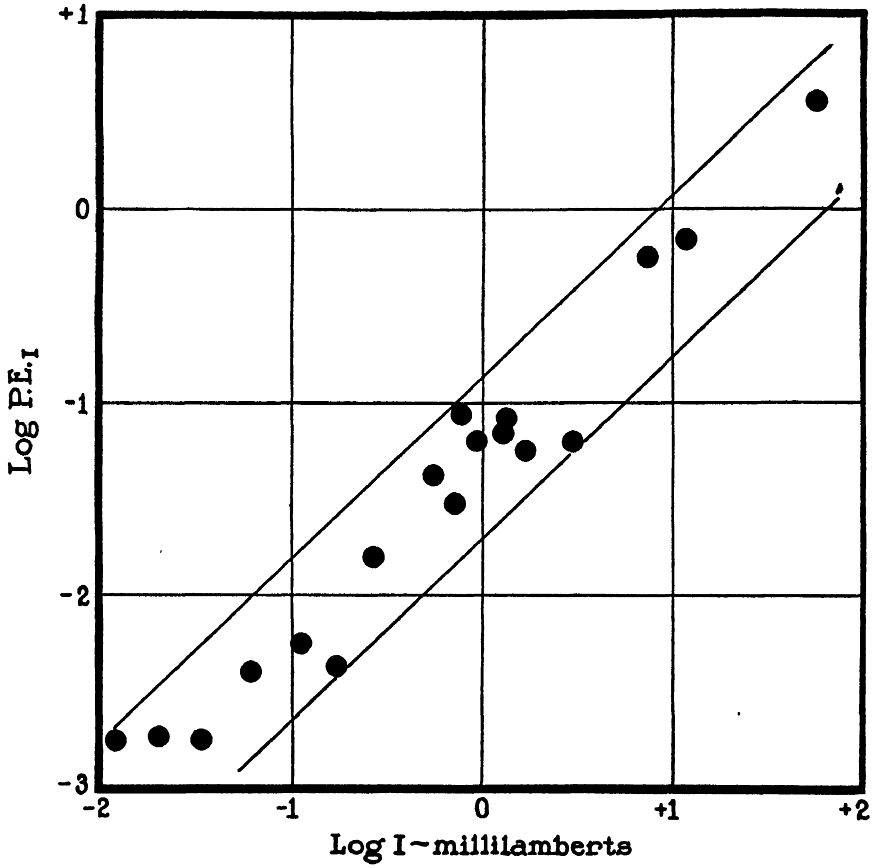

FIG. 4. Relation between the logarithm of the probable error of threshold intensity I and light intensity at which a response of the bee is obtained.

SUMMARY

The bee's characteristic response to a movement of its visual field is used for the study of the relation between critical frequency of flicker and illumination. The critical flicker frequency varies with illumination in such a way that with increasing flicker frequency the intensity of illumination must be increased to produce a threshold response in the bee.

The illuminations required to give a response in a bee at different flicker frequencies closely correspond to the intensities for threshold response in visual acuity tests. This is due to the different thresholds of excitability of the elements of the ommatidial mosaic.

An analysis of the variation of the values for threshold intensities at the several flicker frequencies shows that the variation depends upon flicker frequency and upon the number of elements functioning at different intensities.

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protein or other material into equilibrium with a definite partial pressure of water vapor than to obtain a uniform sample of definite water content.

2. Whether or not the process of denaturation is assumed to be essentially a reaction between protein and water, it is obviously desirable to have a measure of water concentration or activity. The relative humidity (p/p_s) is the most suitable quantity for this purpose.

3. The results are in a form which may be directly compared with analogous experiments on the death rate of living organisms in atmospheres of various relative humidities. It is, of course, in general impossible to determine the actual quantity of water taken up by microorganisms.

In addition, however, we have determined the actual water content of the heated and unheated egg albumin corresponding to each value of the relative humidity, so that it is also possible to relate the denaturation temperature directly to that quantity. The necessary experimental data are given in the last section of this paper.

Technique

The technique employed was similar to that used by Lewith. Dry, crystallizable egg albumin was prepared by drying salt-free solutions over P_2O_5 in an electric refrigerator. The resulting material is white, readily soluble in distilled water except for a trace ($<$ 2 per cent), and is capable of being recrystallized from $(NH_4)_2SO_4$ solution. Samples of this dry protein were placed in desiccators at room temperature over saturated salt solutions giving a wide range of relative humidities.

When the protein had come into equilibrium (as determined by constancy of weight) with water vapor at each relative humidity, three or four samples from each desiccator were placed in small tubes, sealed, and blown into a small bulb at one end. These tubes were made of 0.3 cm. thin walled glass tubing and were originally about 3.75 cm. long and of 0.25 cc. internal volume. The sample placed in each tube weighed from 5 to 10 mg. The open tubes were replaced in the desiccators from which the respective samples had been withdrawn and were left there for several days in order to insure continued equilibrium between protein and water vapor from the saturated salt solutions. Finally, the tubes were quickly sealed at such a distance as not to affect the egg albumin. The glass immediately in contact with the protein never became so warm that it could not comfortably be held. Samples were repeatedly tested and found to possess the same solubility as the original material.

The tubes of native egg albumin in equilibrium with atmospheres of various relative humidities (p/p_s) so obtained were heated in an air thermostat at several temperatures to determine the range for each relative humidity in which the protein neither became completely insoluble nor remained entirely soluble in a definite time interval. Solubility was determined by observing the behavior of particles of the protein in a drop of distilled water on a microscope slide. Because of the extremely high temperature coefficient of denaturation, the range can be easily determined to within less than 10°C. The centers of the ranges determined for the various relative humidities are taken to correspond approximately to temperatures of equal denaturation velocity.

EXPERIMENTAL RESULTS

Tables I and II give the experimental data corresponding to heating times of 10 minutes and 60 minutes, respectively.

The data are summarized in Table III and presented graphically in Fig. 1.

It will be observed from Fig. 1 that the temperature of denaturation is very closely a linear function of the relative humidity. The points referring to p/p_s = 0 per cent and a heating time of 60 minutes, and to p/p_s = 20 per cent for both heating times fall furthest from the straight line. The latter deviation is undoubtedly due to the fact that the potassium acetate solution had not remained saturated, and therefore the humidity must have been too high. This fact also explains the apparent high value for the sorption of water at this relative humidity (see below). The former deviation (at p/p_s = 0 per cent) cannot be explained in this manner. According to the statement of Wichmann, thoroughly dry egg albumin remains soluble after heating at 150°C. for several hours. In our experiments, however, we could never obtain soluble protein at so high a temperature even after 1 hour. Several months after the main series of experiments was carried out, we repeated the tests at p/p_s = 0 per cent with egg albumin samples which were dried with special care over P_2O_5. The temperature of denaturation was essentially the same as that previously found.

DISCUSSION

The experimental relation between relative humidity and the temperature of denaturation may be reduced to a more easily comprehensible form by assuming that the Arrhenius equation

$$\ln k = \frac{Q}{RT} + \text{constant}$$

TABLE I

Dependence of Temperature of Denaturation upon Relative Humidity. Time of Heating = 10 Minutes

Initially in equilibrium with	p/p_0 20°C.	Temperature of thermostat	Solubility*	Range of partial denaturation	Mid-point of range
	per cent	°C.		°C.	°C.
P_2O_5	0	136.0–136.5	+++		
“	“	153 –154	+++		
“	“	158 –159	++−		
“	“	166 –167	−−−		
“	“	168 –169	−−−	158–166	162
$LiCl \cdot H_2O$ (saturated solution)	15	136.0–136.5	+++		
“ “ “	“	145 –146	+++		
“ “ “	“	153 –154	−−−		
“ “ “	“	166 –167	−−−	146–153	150
KAc (saturated solution)	20	128 –129	+++		
“ “ “	“	130.5–131.5	+++		
“ “ “	“	135 –136.5	−−−	130.5–135	133
$CaCl_2 \cdot 6H_2O$ (saturated solution)	32	113	+++		
“ “ “	“	114.5–115.5	+++		
“ “ “	“	121.5–122.5	+++		
“ “ “	“	127 –128	+±−		
“ “ “	“	131 –132	−−−	125–131	128
KCNS (saturated solution)	47	105 –106	+++		
“ “ “	“	108.5–109.5	+−−		
“ “ “	“	111.5–113	−−−	106–110	108
$NaBr \cdot 2H_2O$ (saturated solution)	58	90 – 91	+++		
“ “ “	“	95	+++		
“ “ “	“	97 – 98	+++		
“ “ “	“	100	+++		
“ “ “	“	104.5–105.5	+−−	100–106	103
$NaClO_3$ (saturated solution)	75	81 – 82	+++		
“ “ “	“	84	+++		
“ “ “	“	91.5–92.5	−−−		
“ “ “	“	95	−−−		
“ “ “	“	97 – 98	−−−	85– 91	88

TABLE I—*Concluded*

Initially in equilibrium with	p/p_s 20°C.	Temperature of thermostat	Solubility*	Range of partial denaturation	Mid-point of range
	per cent	°C.		°C.	°C.
KBr (saturated solution)..........	84	73 - 74	+++		
" " "	"	78 - 79	++−		
" " "	"	82	−−−		
" " "	"	90 - 91	−−−	75- 81	78
$NH_4H_2PO_4$ (saturated solution)....	93	65 - 65.5	++−		
" " "	"	69.5- 70.5	+−−		
" " "	"	73 - 73.5	−−−	65- 71	68
H_2O..........................	Saturated	65 - 65.5	+±−		
"	"	69.5- 70.5	±−−		
"	"	73 - 73.5	−−−	64- 71	67.5

* The pluses and minuses indicate the approximate solubility of the heated egg albumin in distilled water.

applies.[1] This assumption is justified since the Arrhenius equation is known quite generally to describe the dependence of the velocity of chemical reactions upon temperature. In particular, the denaturation of egg albumin in dilute solution follows this equation (Lewis, 1926).

An experimental relation which greatly facilitates the application of the Arrhenius equation is that the temperature coefficient (and the critical increment, also) of the denaturation reaction is, within the limits of error, independent of the relative humidity. This is merely another way of expressing the fact illustrated in Fig. 1 that the denaturation temperature: relative humidity curves, corresponding to heating times of 10 minutes and 60 minutes, are very closely parallel. It also follows, of course, that the reciprocal of the absolute temperature of denaturation is a linear function of p/p_s and the curves for the two heating times are parallel.

By means of the Arrhenius equation it is possible from a knowledge of the relative velocities of the denaturation process at the same relative humidity but at two different temperatures (Fig. 1) to calculate

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[1] The author wishes to express his appreciation to Professor E. A. Guggenheim for pointing out this method of interpreting the experimental data.

TABLE II

Dependence of Temperature of Denaturation upon Relative Humidity. Time of Heating = 60 Minutes

Initially in equilibrium with	p/p_0 20°C.	Temperature of thermostat	Solubility*	Range of partial denaturation	Mid-point of range
	per cent	°C.		°C.	°C.
P_2O_5	0	133 -134	+++		
"	"	139 -140	+±−		
"	"	144 -145	−−−		
"	"	149 -150	−−−	137-143	140
LiCl (saturated solution)	15	128.5-130	+++		
" " "	"	133 -134	+±−		
" " "	"	140 -141	−−−	131-137	134
KAc (saturated solution)	20	120 -121	+++		
" " "	"	124 -125.5	+±−		
" " "	"	125 -126.5	−−−		
" " "	"	128 -129	−−−	122-126	124
$CaCl_2$ (saturated solution)	32	111 -113	+++		
" " "	"	118 -119	+++		
" " "	"	120 -121	++−		
" " "	"	124 -125	−−−		
" " "	"	125 -126.5	−−−	119-123	121
KCNS (saturated solution)	47	90.5- 91.5	+++		
" " "	"	95 - 96	+++		
" " "	"	99 -101	+−−		
" " "	"	111 -113	−−−	96-102	99
$NaBr \cdot 2H_2O$ (saturated solution)	58	86 - 87	+++		
" " "	"	90.5- 91.5	++−		
" " "	"	95 - 96	−−−		
" " "	"	99 -101	−−−	90- 96	93
$NaClO_3$ (saturated solution)	75	70.0- 70.5	+++		
" " "	"	74 - 75	++−		
" " "	"	80 - 81	±−−		
" " "	"	86 - 87	−−−	73- 81	77
KBr (saturated solution)	84	59.0- 59.5	+++		
" " "	"	65 - 65.5	+±−		
" " "	"	70.0- 70.5	−−−		
" " "	"	74 - 75	−−−	62- 68	65
$NH_4H_2PO_4$ (saturated solution)	93	51.5	+++		
" " "	"	56	++−		
" " "	"	59 - 59.5	±−−		
" " "	"	65 - 65.5	−−−	54- 60	57

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* The pluses and minuses indicate the approximate solubility of the heated egg albumin in distilled water.

the relative velocity at any other temperature. This can be done for every experimentally determined temperature of denaturation corresponding to every relative humidity. Actually, absolute values of denaturation velocity have not been calculated but only the logarithms of the relative velocities referred to the velocity at 100°C. and $p/p_s = 58$ per cent as unity.

TABLE III

Summary of Data for Dependence of Temperature of Denaturation upon Relative Humidity

p/p_s	Temperature of half denaturation in 10 min.	Temperature of half denaturation in 60 min.
per cent	*°C.*	*°C.*
0	162	140
15	150	134
20	133	124
32	128	121
47	108	99
58	103	93
75	88	77
84	78	65
93	68	57

Fig. 2 illustrates the remarkable fact that the logarithms of the relative velocities at any definite temperature are a strictly linear function of the relative humidity, *i.e.*

$$\ln k = a \cdot p/p_s + b$$

or

$$k = c \cdot e^{ap/p_s}$$

where k is the denaturation reaction velocity constant, e is the base of the natural system of logarithms, p/p_s is the relative humidity, c is a constant dependent upon the temperature but independent of the relative humidity, and a and b are constants independent of both of these variables.

This is the first time to our knowledge that an exponential relation has been observed between the velocity of a chemical reaction and the concentration of one of the reacting substances. Although it is evident that the concentration of water is of extreme importance in determining the velocity of denaturation, it is difficult to imagine what is the physical meaning of the exponential law. A possible line of explanation is that the variation of the relative humidity not only alters the concentration and activity of the water, but also affects

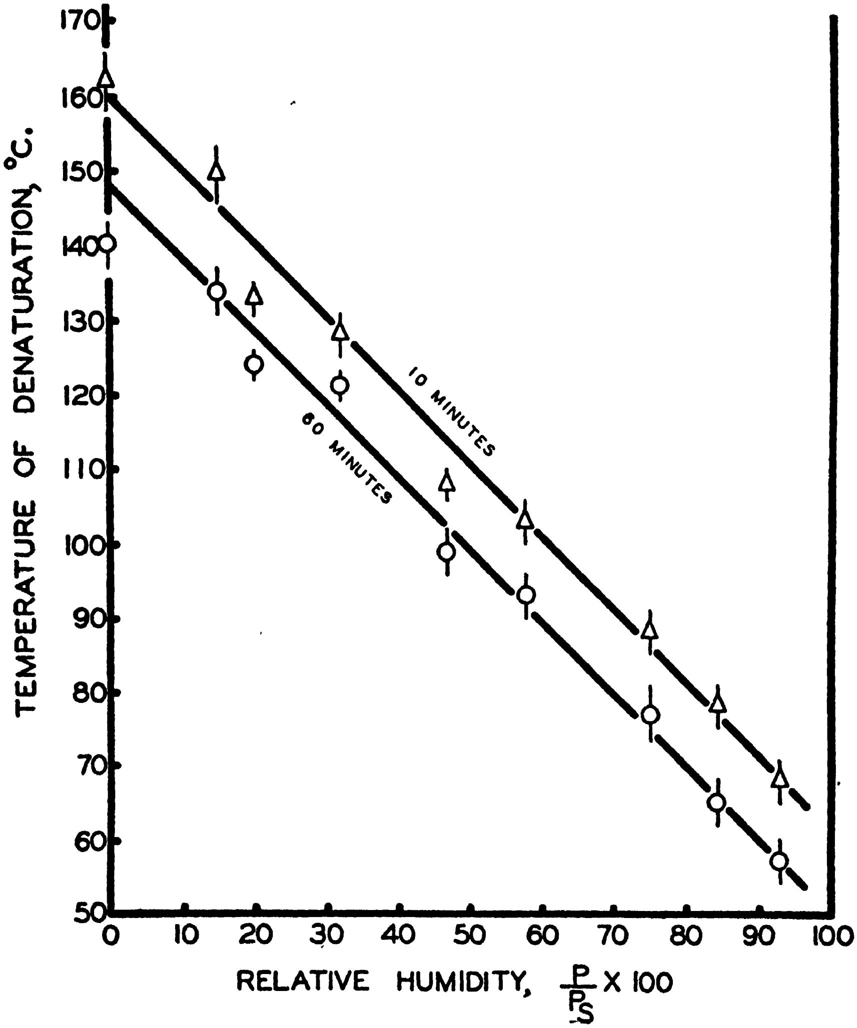

Fig. 1. The dependence of the temperature of denaturation of egg albumin upon the relative humidity of water vapor. △—experimental heating time 10 minutes; ○—experimental heating time 60 minutes.

the freedom of the water molecules to move between and upon the relatively immobile protein molecules and aggregates. The relative immobility of the water at low vapor pressure may be even more im-

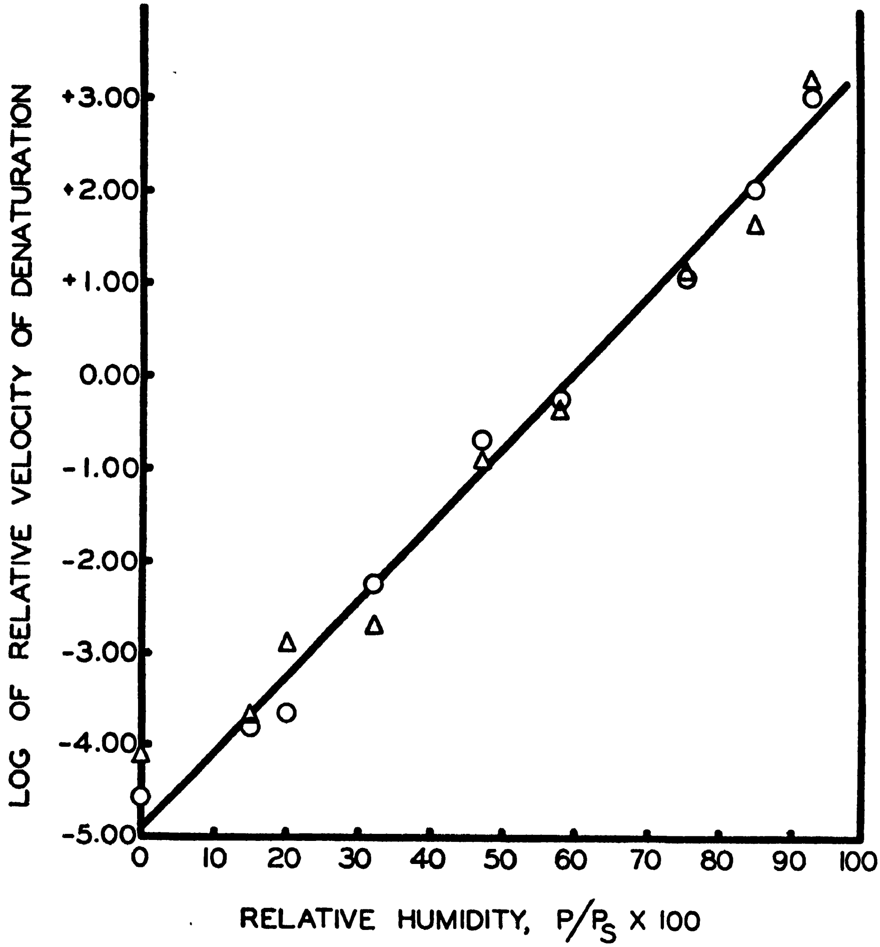

FIG. 2. Showing that the logarithm of the velocity constant of heat denaturation is a linear function of the relative humidity. ○—experimental heating time 10 minutes; △—experimental heating time 60 minutes.

portant in lowering the denaturation velocity than is the reduction in water concentration.

We have assumed that water actually reacts with the protein in the heat denaturation process. It must be admitted, however, that our observations give no conclusive proof that this is true. It is conceivable that the indirect influences which we have just discussed can account for the entire importance of the water. Nevertheless it seems highly probable that the rôle of the water is also a direct one. These kinetic experiments must be considered to support this hypothesis.

We wish to point out that the observed relation between the temperature of denaturation and relative humidity affords a very direct method of studying the mechanism of thermal death in partially dried microorganisms such as bacterial spores and protozoan cysts. The effect of humidity is so great that its influence upon thermal death rate may be easily studied even in the presence of disturbing factors. And if it could be shown that the same relation also applies to this phenomenon, then that would constitute important evidence in support of the hypothesis that thermal death of dry microorganisms is due to protein alteration. Such experiments are in progress.

Water Vapor Pressure Isotherms of Native and Heat-Denatured Egg Albumin

As mentioned above, we have determined the water content of egg albumin as a function of the relative humidity over the entire range. These data are useful not only as a means of converting relative humidities into water contents and *vice versa*, but they also constitute a contribution to the question, so frequently discussed, of the gain or loss of water accompanying heat denaturation.

The sorption isotherms were determined by the desiccator method. Approximately 1 gm. of dry but soluble and crystallizable egg albumin was placed in each of thirty small weighing bottles. These were divided into two groups of fifteen each, one group being heated in a pressure steam sterilizer at 15 pounds per sq. inch for 10 minutes to give the denatured egg albumin, while the other group remained at room temperature. Half of the bottles of each group were brought to constant weight over P_2O_5 (p/p_s = 0 per cent), the other half of each group being similarly brought to equilibrium over a solution saturated with $NH_4H_2PO_4$ (p/p_s = 93 per cent). After this initial equilibrium had been attained, sorption experiments were carried out with one set of bottles by placing them in vacuum desiccators over solutions of higher relative humidities, while the other set was placed at lower relative humidities for desorption experiments. In all experiments the anhydrous weight of the protein was determined as the final constant weight obtained over solid P_2O_5 which will undoubtedly agree closely with Sørensen and Høyrup's (1917) anhydrous protein dried to constant weight over solid potassium hydroxide.

1 or 2 weeks were required to attain constant weight. The change of weight was followed by weighings made on an analytical balance every 1 to 4 days. As soon as the desiccators were opened for weighings, the ground glass stoppers were placed on the weighing bottles contained therein.

We shall especially mention that the solubility and crystallizability of the dried native egg albumin were tested at intervals throughout the duration of the experiments. Every sample was found to be completely or almost completely ($>$ 98 per cent) soluble in distilled water and almost all were recrystallizable from ammonium sulfate solution.

Table IV gives the experimental data which are represented graphically in Fig. 3.

The experimental data may be summarized by the statement that heat-denatured egg albumin takes up approximately 80 per cent as much water at each particular relative humidity as does native crystallizable egg albumin. This conclusion is in agreement with the less complete experiments of Sørensen and Sørensen (1925), Katz (1917), and Adair and Robinson (1931) as regards both the sign and the magnitude of this effect.

A comparison of the results of these sorption experiments with those from the study of the dependence of denaturation velocity upon relative humidity leads to the apparently anomalous conclusion that whereas water is necessary for denaturation and denaturation itself probably involves a reaction of the protein with water, yet the denatured protein is less heavily hydrated than the material from which it is derived. Even if the kinetic evidence demands the conclusion that the denaturation reaction actually involves a combination of water with the protein, it nevertheless can suggest nothing at all about the properties of the resulting protein. The quantity of water which might react would be too small to be analytically demonstrable

TABLE IV

Sorption of Water by Native and Denatured Egg Albumin. Temperature Range, 20–26.75°C. Mean Temperature, 23°C. x/m in Gm. Per Gm. of Anhydrous Protein

Protein in equilibrium with saturated solution of	p/p_0 20°C.	Sorption		Desorption		Mean x/m		Ratio b/a
		Native	Denatured	Native	Denatured	Native (*a*)	Denatured (*b*)	
	per cent							*per cent*
$LiCl \cdot H_2O$	15	0.0376		0.0453	0.0400			
				0.0455	0.0377			
				0.0386	0.0294			
					0.0388	0.0417	0.0365	87
KAc	20	0.0612		0.0682		0.0647		
$CaCl_2 \cdot 6H_2O$	32	0.0748		0.0839	0.0643			
				0.0778	0.0550			
				0.0866	0.0604	0.0808	0.0599	74
KCNS	47	0.0974		0.1067		0.1021		
$NaBr \cdot 2H_2O$	58	0.1183	0.0957	0.1235	0.1031			
		0.1260	0.1009	0.1310	0.1054	0.1247	0.1013	81
$NaClO_3$	75	0.1637	0.1419	0.1744				
		0.1695	0.1223					
		0.1722	0.1270					
			0.1378			0.1699	0.1322	78
KBr	84	0.2058	0.1532	0.2102	0.1607			
		0.1998			0.1788			
					0.1730	0.2053	0.1664	81
$NH_4H_2PO_4$	93	0.3143	0.2396					
		0.3093	0.2259					
		0.3078						
		0.3006						
		0.3046						
		0.3059						
		0.3061						
		0.3090						
		0.2961				0.3059	0.2327	76
H_2O	Saturated	0.7495	0.3523					
		0.7237	0.3670			0.7366	0.3596	
Mean								79.5 per cent
Mean deviation								3.5 per cent

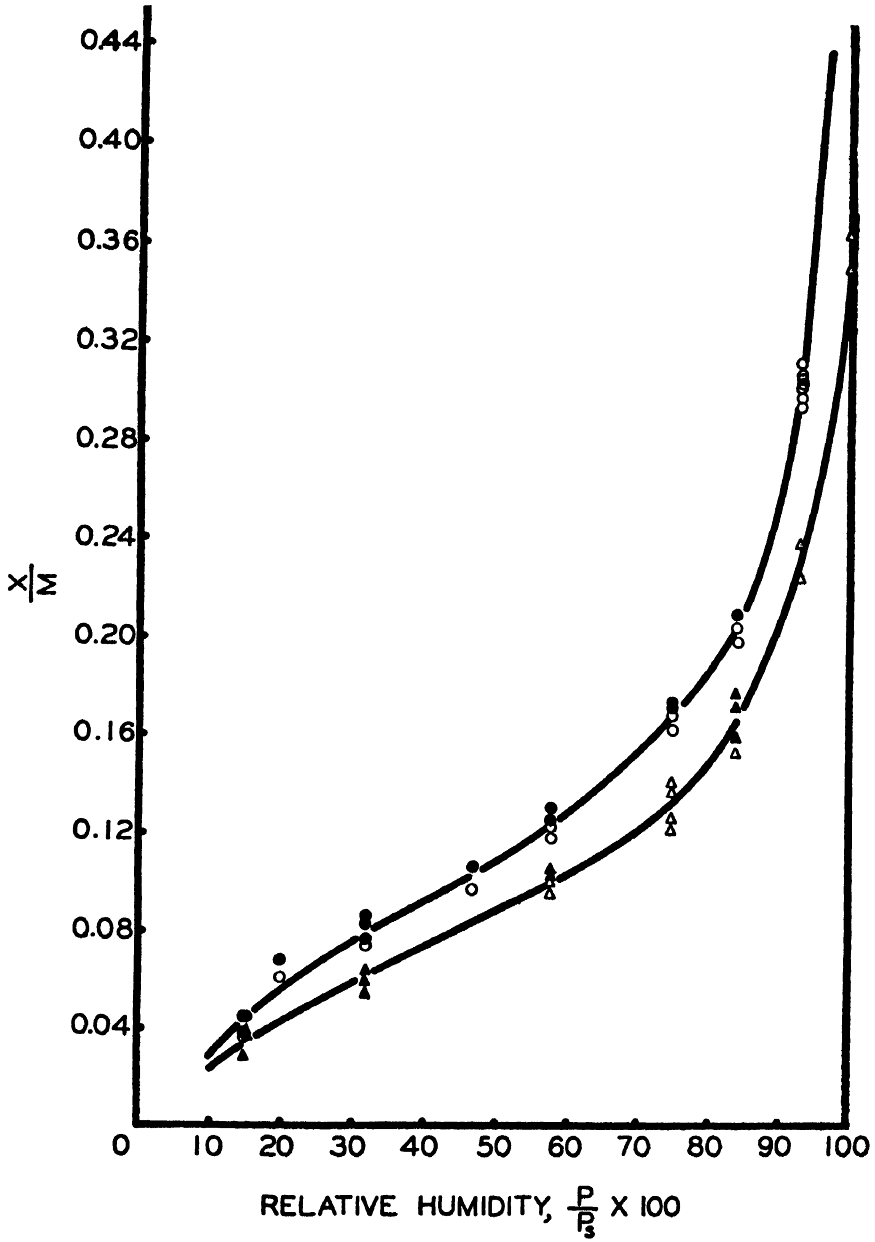

Fig. 3. Sorption of water by native and heat-denatured egg albumin. ○—sorption, native; ●—desorption, native; △—sorption, heat-denatured; ▲—desorption, heat-denatured.

(Hirsh-Pogany, 1922), and would be, moreover, entirely masked by the lesser affinity of the new protein derivative for water.

SUMMARY

1. The denaturation rate of partially dried crystallizable egg albumin is greatly decreased by decreasing its water content.

2. The temperature of denaturation, defined as the temperature at which half of the protein becomes insoluble in distilled water after a definite time of heating, is a linear function of the relative humidity with which the protein is in equilibrium.

3. By applying the Arrhenius equation it is shown that the rate of heat denaturation at a given temperature is an exponential function of the relative humidity.

4. The application of the observed relations to the analysis of the mechanism of thermal death of microorganisms is suggested.

5. The water content of native and heat-denatured egg albumin is determined as a function of the relative humidity of water vapor. It is shown that the heat-denatured modification takes up approximately 80 per cent as much water at all relative humidities as does native egg albumin.

In conclusion the author wishes to express his appreciation to Professor J. W. McBain for advice and criticism during the progress of this work.

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GELATINASE AND THE GATES-GILMAN-COWGILL METHOD OF PEPSIN ESTIMATION

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(Accepted for publication, June 15, 1933)

The convenience of the Gilman and Cowgill development (5) of the Gates technique (3, 4) for the estimation of proteolytic power has encouraged its wide adoption. This method is photometric and depends on the decrease in opacity of a completely developed photographic film after treatment with a solution of a protease. The principle involved here is the partial solution of the gelatin which holds the particles of completely reduced silver. The writer has used this method in over 12,000 determinations on canine gastric juice. Northrop's conclusion (6) that crude pepsin contains a specific gelatin-liquefying enzyme ("gelatinase"), in addition to crystallizable pepsin, made it advisable to ascertain whether the method really measures the activity of pepsin, of gelatinase, or of both.

Northrop (6) distinguished gelatinase from pepsin in part by the greater resistance of the former to alkali: whereas pepsin was completely inactivated at pH values above 9, gelatinase was still more than 50 per cent active at that alkalinity, and retained some effectiveness even at values above 10. Advantage was taken of this difference to test qualitatively for gelatinase activity in both the commercial pepsin standard (Armour and Co., U. S. P. 1:10,000) and in canine gastric juice.

For these experiments a series of standard buffers was prepared ranging from an acidity of approximately pH 1 to an alkalinity near 13. The series chosen was that of Sörensen, described by Clark (2, pp. 203–210); glycine-NaCl-HCl, glycine-NaCl-NaOH and citric acid-NaOH were the three series selected. The hydrogen ion concentrations of all these solutions were determined by a hydrogen electrode in a cell whose liquid junction potential was assumed to be eliminated by the insertion of a saturated KCl bridge. The cell used was:

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* A part of these data are contained in the dissertation presented by Elizabeth R. B. Smith to the Faculty of the Graduate School, Yale University, May, 1933, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

$$\text{Pt, } H_2/\text{buffer/saturated } KCl/HgCl/Hg.$$

In calculating the pH values, the equations recommended by Clark (2) were used.

In these tests, three series of "peptic activity" estimations were made under the usual conditions of the Gilman-Cowgill method (5) except for the hydrogen ion concentrations of the buffers in the cells. Series A was a blank, buffer only being placed in the cells. Series B contained a 0.5 per cent concentration of the 1:10,000 pepsin normally used as a standard in the determination. Series C held a mixture of canine gastric juice samples diluted in each case with four parts of the buffer solution. The results obtained are best expressed by Table I. The unitage of "pepsin" recorded was calculated from a standard line set by a dilution series run in the usual way on films of the same preliminary reading as those of the experimental series. For a more detailed explanation of this the reader is referred to Gilman and Cowgill's description of the method (5). Here, however, 1 per cent of the 1:10,000 commercial pepsin represents 100 units per cc. instead of 1000 units, the value adopted by Gilman and Cowgill.

By reference to Table I it can be seen that above pH 8.5 no proteolytic power was evinced. The change in opacity of the films of Series B and C at these higher pH values was no greater than that of the more alkaline members of Series A. The results were the same whether the solutions were made up 24, 12, or 2 hours prior to the running of the series.

At the suggestion of Dr. J. H. Northrop, further experiments were made to determine more exactly the value of the gelatin film method in the measure of the peptic or gelatinase activity of protease solutions. Dr. Northrop kindly furnished us with three solutions whose activity had been determined by various methods including the hemoglobin technique of Anson and Mirsky (1) and the gelatin viscosity method of Northrop and Hussey (8) as later modified by Northrop (7). These three test solutions may be described as follows: Solution P D, 0.8 per cent commercial pepsin containing 0.022 unit per cc. by the hemoglobin technique and 2.1 units per cc. by the gelatin viscosity method; Solution C, four times recrystallized pepsin testing as 0.022 unit by the hemoglobin procedure and 1.4 units per cc. by the gelatin viscosity technique; and Solution G, a crude preparation of gelatinase corresponding approximately to the fraction P_4 described by Northrop (6), and containing 0.022 hemoglobin unit and 7 gelatin units per cc.

TABLE I

Buffer pH*	Peptic activity in units per cc.		
	Buffer only. Series A	0.5 per cent pepsin. Series B	Diluted canine gastric juice. Series C
1.05	—	53	120
1.44	—	55	114
2.12	—	50	115
2.46	—	42	100
2.78	—	43	100
3.20	—	40	96
3.58	—	37	91
4.01	—	30	75
4.40	—	26	69
4.51	—	28	70
5.04	—	20	50
5.58	—	18	42
6.02	—	10	21
6.74	—	5	11
8.47	—	Trace	?
9.22	2	—	—
9.95	3	2	4
10.30	—	3	?
11.10	5	4	3
11.88	3	3	3
12.17	2	5	5
12.64	2	3	2
12.75	3	3	3

* These values were not materially affected by the additions of commercial pepsin or of small quantities of gastric juice.

These three solutions were examined by the Gilman and Cowgill technique both at the usual reaction recommended for the determina-

TABLE II

Material	Method	pH	Temperature	Units per cc.
Solution P D	Hemoglobin	—	—	0.022*
0.8 per cent commercial pepsin	Viscosity	5.0	35.5	2.1*
	“	4.5	34.0	1.8
	Film	1.8	25.0	138.0
	“	2.8	25.0	125.0
	“	3.6	25.0	120.0
	“	4.8	25.0	105.0
	“	8.4	25.0	35.0
Solution C	Hemoglobin	—	—	0.022*
4 × recrystallized pepsin	Viscosity	5.0	35.5	1.4*
	“	4.5	34.0	1.2
	Film	1.8	25.0	135.0
	“	2.8	25.0	135.0
	“	3.6	25.0	133.0
	“	4.8	25.0	103.0
	“	8.4	25.0	20.0
Solution G	Hemoglobin	—	—	0.022*
Gelatinase fraction P_4	Viscosity	5.0	35.5	7.0*
	“	4.5	34.0	6.0
	Film	1.8	25.0	138.0
	“	2.8	25.0	140.0
	“	3.6	25.0	155.0
	“	4.8	25.0	175.0
	“	8.4	25.0	85.0
1 per cent Armour	Viscosity	4.5	34.0	1.7
1:10,000 pepsin	Film	1.8	25.0	100.0 (by definition)
	“	2.8	25.0	92.0
	“	3.6	25.0	90.0
	“	4.8	25.0	95.0
	“	8.4	25.0	20.0
Canine gastric juice	Viscosity	4.5	34.0	3.7
	Film	1.8	25.0	170.0
	“	2.8	25.0	180.0
	“	3.6	25.0	175.0
	“	4.8	25.0	165.0
	“	8.4	25.0	50.0

* Value furnished by Dr. J. H. Northrop.

tion and at more alkaline pH values. The buffers used were similar to but not identical with those used in the preceding experiments and were also checked electrometrically with respect to hydrogen ion concentration. A commercial pepsin and canine gastric juice were estimated at the same time by both the gelatin film method and the gelatin viscosity technique. In the latter determination, the conditions defined by Northrop and Hussey (8) rather than those set up by Northrop (7) were used; the unitage, however, was calculated by the method of Northrop (7). The difference in conditions no doubt accounts for the failure to check exactly the units as found by Northrop; it should be noted, however, that the values found by us are in each case exactly six-sevenths of the figures furnished us by Dr. Northrop. The findings from this later set of experiments are set forth in Table II.

It is apparent from these figures (Table II) that the Gates method measures the same enzyme or enzymes as found by the hemoglobin method. Therefore it would seem that the film method determines chiefly pepsin, though no doubt gelatinase is a factor in some cases and if the pH used were higher might interfere markedly.

The following conclusions can be drawn from the results of Tables I and II: The enzyme activity measured, as has been said, is the same by the gelatin film method as by the hemoglobin technique of Anson and Mirsky. The unitage of each sample as determined by the Gates procedure checks closely with the hemoglobin figures and is not in accord with the values as estimated by the gelatin viscosity method. The higher values obtained with the latter method on the canine gastric juice sample are interesting; they suggest the presence of some gelatinase activity in this material. This was pure gastric juice collected from a Pavlov gastric pouch in response to the ingestion of food. This finding should be followed and checked.

The conclusion to be drawn with regard to the validity of the Gates method for pepsin estimation is that this gelatin film method constitutes as nearly a truly peptic method as does any other extant. It is not influenced by gelatinase as is the gelatin viscosity estimation. The findings at lower acidities shown in Table II suggest that the extremely low (almost physiological) pH, as well as perhaps the comparatively low temperature (25°C.), may be a factor in this in view of the fact that the comparative potency of the gelatinase increased as the pH approached 5, that used in the gelatin viscosity method.

SUMMARY

The Gates photographic film method for pepsin estimation as developed by Gilman and Cowgill measures an activity corresponding to that determined by the hemoglobin method of Anson and Mirsky rather than that resulting from the use of the gelatin viscosity technique. Therefore, the presence of gelatinase is not a source of great error in the gelatin film procedure.

The writer wishes to acknowledge her indebtedness to Dr. J. H. Northrop of The Rockefeller Institute for his generosity in furnishing the crystalline pepsin and gelatinase solutions used, and to Dr. D. I. Hitchcock of the Department of Physiology and Dr. Raymond Hussey and Dr. W. R. Thompson of the Department of Pathology, Yale University School of Medicine, without whose courteous loans of materials and apparatus these experiments would not have been possible.

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THE REFRACTIVE INDICES OF WHOLE CELLS

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I

To deal quantitatively with the distribution of light within the sporangiophore of *Phycomyces*, the constants of this optical system must be known. The following measurements were made in order to obtain a value of the refractive index of the cells.

It is evident that a cylindrical cell with relatively transparent and homogeneous contents will function in air as a converging lens. The radius of curvature is one-half of the cell diameter, which may be measured directly with a microscope having an ocular micrometer. The refractive index of the intact cell may be determined by allowing parallel light to fall on the cell and measuring the focal distance. This method was originally applied to cellular constituents by Nägeli and Schwendener (1867), later to intact plant cells by Senn (1908), and to developing sea urchin eggs by Vlès (1921).

The focal distance which is measured with intact cells results from the combined action of all cellular constituents, which may not have the same refractive indices. According to Oort and Roelofsen (1932) the chitinous wall of the sporangiophore of *Phycomyces* reaches a maximum thickness of 3 μ below the growing zone, and in the growing zone is less than 1 μ thick. The diameters of the sporangiophores used in these measurements ranged from 48 to 120 μ. Because of the extreme thinness of this wall no large error is introduced by leaving it out of consideration, although it certainly has a higher refractive index than the other cell constituents. At the growing zone, the sporangiophore appears completely filled with a homogeneous mass of "protoplasm." In all other regions there is a central sap vacuole which often occupies one-half of the total diameter of the cell. Focal distances were measured both at and below the growing zone. Since calculations of the refractive indices in these two regions show no significant difference, it is evident that the presence or absence of a central sap vacuole introduces an error which is within the precision of the method. In consideration of sources of error which will be discussed below, it seems justifiable to treat the sporangiophore as a homogeneous cylinder of protoplasm in computing the effective refractive index.

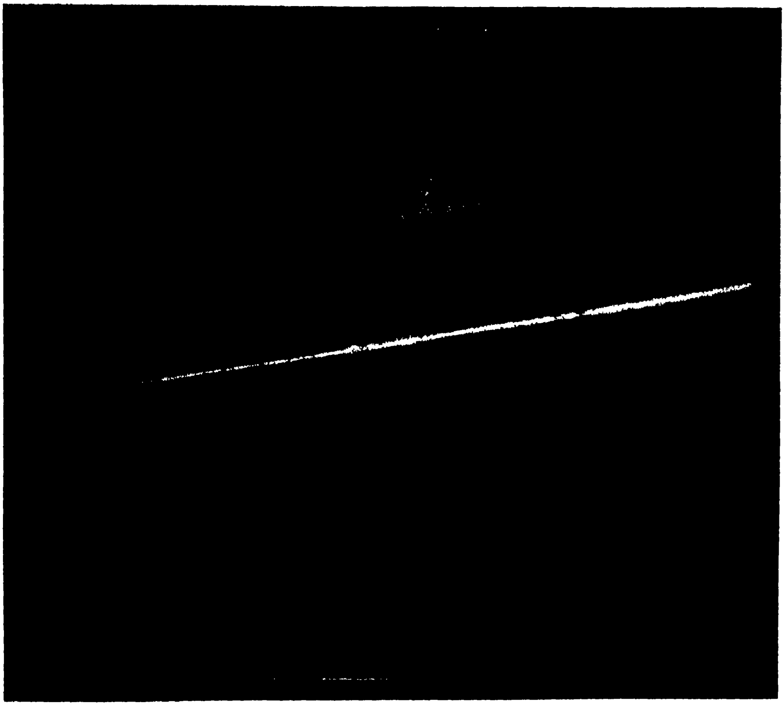

FIG. 1. Photomicrograph of the "bright line" to which parallel light incident from below is converged by a cell of *Phycomyces* in air. The line is about 0.04 mm. above the upper cell wall, hence no part of the cell itself is in focus. Approximately ×375.

Growing sporangiophores were placed horizontally on the stage of a microscope, and supported at the free end to prevent sagging. At a distance of 1 meter was a clear, concentrated filament, 500 watt, 115 volt projection bulb, which served as a source of approximately parallel light. A beam of light was allowed to strike the plane substage mirror of the microscope, and was reflected vertically upward to the sporangiophores. The substage condenser was removed. Looking through the microscope, a sharp bright line could be brought into focus slightly above each cell (see Fig. 1). By means of the fine adjustment, the microscope tube was then lowered until particles streaming in the topmost peripheral layer of protoplasm came into focus. The level of these moving particles could be located more consistently than the top of the cell wall or the middle plane of the cell. While the locus of this middle plane is what is desired for determination of the focal distance, it could not be focused upon with any degree of accuracy. The vertical distance through which the tube moved was equal to the difference between the readings of the fine adjustment micrometer in the two positions, the micrometer drum being graduated in single μ.[1] Each distance was determined by separate settings for each cell ten times. The precision of setting was greatest with rather high powers of magnification (objective – 45×; ocular – 6×).

Due to the high intensity of light needed for the measurements, a monochromatic source could not be used. Measurements made with white light are probably best referred to the spectral region to which the eye is most sensitive (approximately 500 mμ). On this assumption, the index of refraction with reference to the sodium line (589.3 mμ) would be not more than 1 unit in the second decimal place less than the value obtained. The measurements were carried out at a room temperature of 20–22°C.

II

The focal distance of a cylindrical lens placed in parallel light is the distance along the axis from the center of the lens to the spot where the rays converge to a "point." In the procedure described above, not the center of the cell but a point immediately within the cell wall was determined. In order to find the center, a distance equal to the radius of the cell minus the thickness of the wall must be added.

If

f = focal distance
f' = measured distance from image to peripheral layer of protoplasm
D = diameter of cell
b = thickness of cell wall

then

$$f = f' + \frac{D}{2} - b \qquad (1)$$

For each cell the radius was known through direct measurement of the diameter. The thickness of the wall was taken as 3μ (*cf.* Oort and Roelofsen, 1932).

As a consequence of spherical abberation in a lens of such wide aperture, all parallel rays do not converge to a point behind it. Hence the sharpest image which can be focused upon is a bright line of finite width, corresponding to the "least circle of abberation" of a spherical lens, which lies slightly nearer the lens than the ideal focal point. The magnitude of the correction of the apparent focal distance for this error was computed on the assumption that the refractive index of the cell was 1.38, the diameter of the image being estimated from photomicrographs as 1.5 μ. The least circle of abberation was found to be probably less than 2 μ nearer the cell than the true focal point. This source of error was therefore disregarded.

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[1] Many microscopes are equipped with fine adjustments containing a lever system, and cannot be used for the precise measurement of vertical distances.

Refractive indices were calculated by means of the following formula for a spherical lens:

$$n = \frac{4fv}{4f - D} \tag{2}$$

where

n = index of refraction of the cell
v = index of refraction of the surrounding medium (air; taken as 1)
f = focal distance
D = cell diameter

Table I gives the measurements and computed indices of refraction for the six cells studied. In each case, the average focal distance is based on ten separate determinations. The extreme values of f obtained for each cell are given. These extremes deviate on the average 5 to 6 per cent from the mean values of f.

It is evident that there is no correlation of refractive index with cell diameter, although the largest cells studied were nearly three times the size of the smallest. The values of n for the six cells may be averaged, yielding a mean value of $n = 1.38$. Since the cell diameters were measured to within 1 or 2 μ, determination of the focal distance introduces the largest single error.

III

Senn (1908) used a similar method for measuring the refractive indices of entire plant cells, but obtained much higher values than those computed here for *Phycomyces*. Table II gives illustrative values from Senn's work.

Since protoplasm is largely composed of water, it is surprising to note Senn's finding of refractive indices for plant cells equal to those for crown glass or paraffin oil. Data on the optical constants of the human eye (von Helmholtz, 1924) also do not accord with Senn's figures. As Table II shows, even the lens of the eye, specialized as it is for refraction of light, has a low index compared with those found by Senn for plant cells. Senn seems to have corrected his measurements for spherical abberation by multiplying the apparent focal length by a constant greater than 1 and increasing with the diameter of the cell. This correction increases the apparent focal distance, and therefore lessens the value of the refractive index. Senn regarded its use as justified by the fact that by this means constant values of

TABLE I

Cell diameter	Minimum focal length	Maximum focal length	Average focal length	Average index of refraction
μ	μ	μ	μ	
48.3	41.5	47.0	44.6	1.37
58.4	50.1	53.1	51.6	1.39
71.2	61.1	69.3	64.3	1.38
90.8	75.0	83.2	79.4	1.40
102.2	90.1	99.1	94.8	1.37
119.1	111.1	117.7	115.4	1.35

TABLE II

Object	*n*	Author
Vaucheria repens (thallus)......................	1.47–1.48	Senn
" *terrestris* (")......................	1.49–1.52	"
Bryopsis plumosa (")......................	1.49–1.52	"
Funaria hygrometrica (paraphyses)................	1.48	"
Phaseolus vulgaris (palisade cells)................	1.47	"
Vicia faba (" ")................	1.49	"
Taraxacum officinale (" ")................	1.48	"
Human eye..................................		von Helmholtz
Cornea..................................	1.376	
Humors..................................	1.336	
Lens (outer layer)........................	1.386	
" (core)................................	1.406	
Sea urchin egg (unfertilized)......................	1.39	Vlès

refractive index could be obtained from cells of different diameters. No such arbitrary correction was used in the work with *Phycomyces*. Cells of widely differing diameter gave comparable values of refractive index, as shown in Table I.

It may be significant that the present measurements were made in air, while all the cells used by Senn were submerged in water. Furthermore, working with spheres of expressed protoplasm, Senn found the same high values of refractive index as for similar, intact protoplasm surrounded by a cellulose wall (n = 1.49). In view of the aqueous nature of protoplasm, this result is almost certainly incorrect. If correct, it would mean that in water cellulose wall and protoplasm could not be distinguished, and this is not true. The method used by Senn must contain a considerable hidden source of error.

Vlès (1921 *a*, *b*) made use of the same method to follow changes in the index of refraction of the sea urchin's egg during early development. He obtained a value of n for the unfertilized egg of approximately 1.393. During cleavage, he recorded rhythmic changes amounting to a deviation of 0.014 from this value. Due to the sequence of form changes of the egg, only one measurement seems to have been made on an egg at any one stage, and the possible influence of the fertilization membrane was disregarded. It is obvious that under these conditions the calculated indices are scarcely accurate to 2 units in the second decimal place. Changes in index of refraction of such eggs may occur during development, but the measurements of Vlès do not substantiate them. His determinations agree, however, in general order of magnitude with those made with *Phycomyces*.

SUMMARY

Refractive indices of intact sporangiophores of *Phycomyces* were computed from measurements of focal length and radius of curvature of the cells. For the six cells studied, effective values of n were obtained ranging from 1.35 to 1.40. The average effective n was 1.38. Senn's determination of refractive indices of other plants cells gave much higher values: n = 1.37 to 1.52. The precision of the method and possible sources of this discrepancy are discussed.

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THE PHYSICAL BASIS OF THE POSITIVE PHOTOTROPISM OF PHYCOMYCES

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I

The measurements described in this paper were made in order to find out how the net photochemical action of light falling on a cell of circular cross-section may be greater in the half of the cell farther from the source of light. It is known that in air the cylindrical, light-sensitive sporangiophore of *Phycomyces* bends toward a single source of light, and that the bending is due to a difference in the rates of growth or extension of the cell wall on opposite sides of the growing zone. With symmetrical illumination of the growing zone there is no bending, but a temporary acceleration of growth, the *light growth* response. The conclusion is therefore unavoidable that when parallel light strikes the sporangiophore from one side, the bending which ensues is caused by relatively more growth, and hence relatively greater photochemical action, on the side farthest from the source of light. Since absorption certainly occurs as light passes through the sporangiophore, how is this possible?

Blaauw (1914) regarded the sporangiophore as a cylindrical lens which, because of its relative transparency, concentrated light on the back wall of the cell in a zone of relatively high intensity. Blaauw and more recently Nuernbergk (1927) were forced to conclude that the brightly illuminated zone on the back wall, even though flanked on both sides by large areas of relative shadow (*cf.* Fig. 1 *A*), served to call forth a greater growth acceleration than was produced at the cell wall nearest to the light. Even neglecting absorption in the cell, this would mean that a certain *amount* of light concentrated in a small area of the cell is more effective than the same amount of light spread over a larger area. If there were small, separate zones of reception and reaction within the cell, this idea might conceivably be correct. The radial symmetry of the sporangiophore and the fact that the region of sensitivity to light coincides with the region of growth do not support the idea. Barring the existence of such cell "organs," explanation of this anomalous action of light in the terms used above is contrary to the whole theory of phototropism as based upon differential photochemical action.

Oehlkers (1926) concluded from inadequate experimental evidence that lens action alone would not explain the positive phototropism of *Phycomyces* in air. He suggested that total reflection at the back wall of rays passing through the peripheral parts of the cell would increase the net absorptive path of light within the far half of the cell. Unfortunately the total reflection diagramed in Oehlkers' paper is physically impossible, as a glance at his incorrect geometrical construction will show.

The solution offered in this paper is based upon the following assumptions: (1) that bending is a consequence of unequal absorption of light in the two halves of the cell; (2) that the primary action of light is on the cell protoplasm rather than on the wall; (3) that the substance which absorbs light is uniformly distributed within the cell.

The first assumption is axiomatic. The second implies that the protoplasm of one half of the cell reacts to light more or less as a whole, at least as regards the production of a differential growth increment. That half of the cell reacts as a whole may merely mean that there is time for the diffusion or transport of photochemical products throughout part of the cell. The latent period of several minutes duration in the response of *Phycomyces* to light may furnish the time necessary for such "summation." In any case, the light growth response of *Phycomyces* is not an immediate, passive stretching of the cell wall under the influence of light.

The third assumption is supported by the observation that the growing zone of the sporangiophore appears filled with an undifferentiated mass of protoplasm, without a central sap vacuole. The protoplasm of this zone moreover contains a yellow pigment. As far as can be judged by eye, this pigment is distributed throughout the protoplasm. The following argument would need only numerical correction were it shown that there is in fact a small central sap vacuole, or that the pigment is mainly located in the peripheral layers of protoplasm.

II

Consider a sporangiophore of *Phycomyces* growing in air and struck by a single beam of parallel light at right angles to the long axis of the cell, as in Fig. 1 *A*. For simplicity, only half of the incident beam is represented. In the following calculations the cell has been treated as an optically homogeneous cylinder of refractive index 1.38 (*cf.* Castle, 1933).

If the cell in Fig. 1 *A* is bisected at right angles to the beam of incident light, as indicated by the dotted line, the relative distance traversed by each ray of light in each half of the cell may be measured on the diagram. Table I gives these measurements for a cell similar to that in Fig. 1 *A*, the cell radius being taken for convenience as 10 mm. If the distances in the two halves be l_1 and l_2 respectively, corresponding to the halves of the cell nearest and farthest from the source of light, then for the central ray only

$$l_1 = l_2$$

For all other rays

$$l_1 < l_2$$

By actual measurement on the diagram, it can be shown that for the whole cell

$$\frac{\Sigma\ (l_2)}{\Sigma\ (l_1)} = 1.32 \text{ (approximately)}$$

Since the density of radiation striking the cell in Fig. 1 *A* is represented by the arbitrary spacing of the parallel lines, it might be supposed that an increase in density would alter the ratio just determined. A more accurate result is obtained by solving graphically for the limiting case, when the density of incident radiation is infinite. Geometrically, this means allowing the distance between the parallel lines to become infinitely small. This distance is measured in angular coordinates by sin i, where i is the angle of incidence of each ray on the cell. Graphic integration should therefore be carried out with respect to sin i, between the limits $\sin i = 0$ and $\sin i = 1$. In Fig. 2 values of l_1 and l_2 from Table I are plotted against sin i. The areas under the

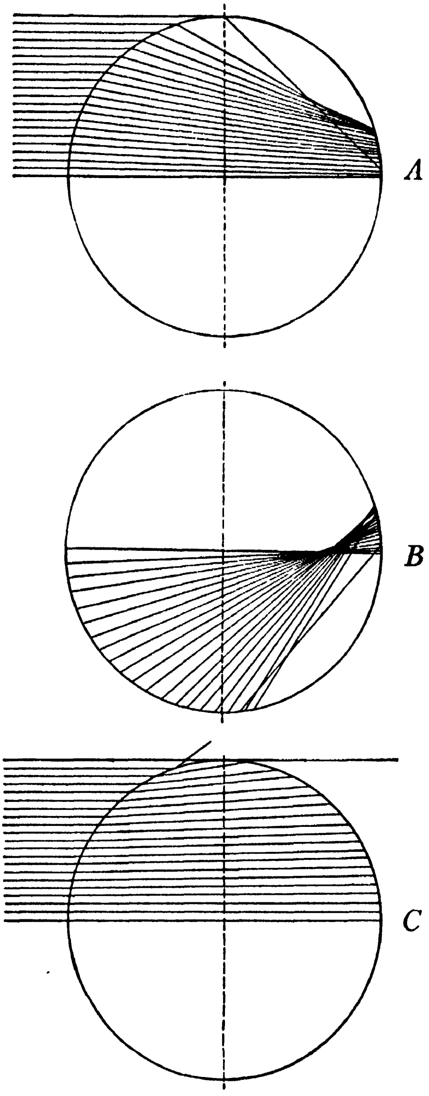

FIG. 1. Diagrams showing light pathways within the sporangiophore of *Phycomyces*. At the growing zone the cell is assumed to be a homogeneous cylinder of protoplasm of refractive index 1.38. For convenience, rays in only one half of the cell are drawn in. *A* — cell in air; *B* — rays of light striking the back wall in *A* are shown here partially reflected back into the cell; *C* — cell in paraffine oil of refractive index 1.47.

TABLE I

Angles of incidence and refraction, loss of intensity by reflection at front wall of cell, and length of path of light rays in the front and back halves of the cell.

Angle of incidence	Intensity loss by reflection	Angle of refraction	l_1	l_2
degrees	*per cent*	*degrees*	*mm.*	*mm.*
0.0	2.6	0.0	10.00	10.00
2.9	2.6	2.1	10.00	10.00
5.4	2.6	3.9	9.98	10.00
7.7	2.6	5.6	9.91	10.00
11.7	2.5	8.4	9.80	10.00
14.6	2.5	10.6	9.71	9.96
17.8	2.5	12.8	9.59	9.94
20.2	2.5	14.5	9.43	9.93
23.8	2.6	17.0	9.25	9.92
26.8	2.6	19.1	9.04	9.89
30.1	2.7	21.3	8.80	9.89
33.6	2.8	23.7	8.50	9.88
37.0	2.9	25.9	8.16	9.85
40.6	3.0	28.1	7.82	9.83
44.5	3.3	30.5	7.39	9.90
48.4	3.8	32.8	6.90	9.99
53.4	4.6	35.6	6.29	10.10
58.3	6.3	38.1	5.62	10.22
64.3	9.6	40.8	4.78	10.45
72.3	18.3	43.7	3.52	11.05
90.0	100.0	46.4	0.00	13.80

The radius of the cell is taken for convenience as 10 mm., the index of refraction as 1.38. l_1 and l_2 were measured on the original of Fig. 1 *A*.

two curves measure the summated distances traversed in the respective quadrants of the cell.

$$\frac{\int_0^1 l_2\, d \sin i}{\int_0^1 l_1\, d \sin i} = \frac{\text{Area under Curve } l_2}{\text{Area under Curve } l_1} = 1.26$$

In other words, the total absorptive path is 1.26 times longer in the half of the cell farthest from the source of light, due to refraction alone.

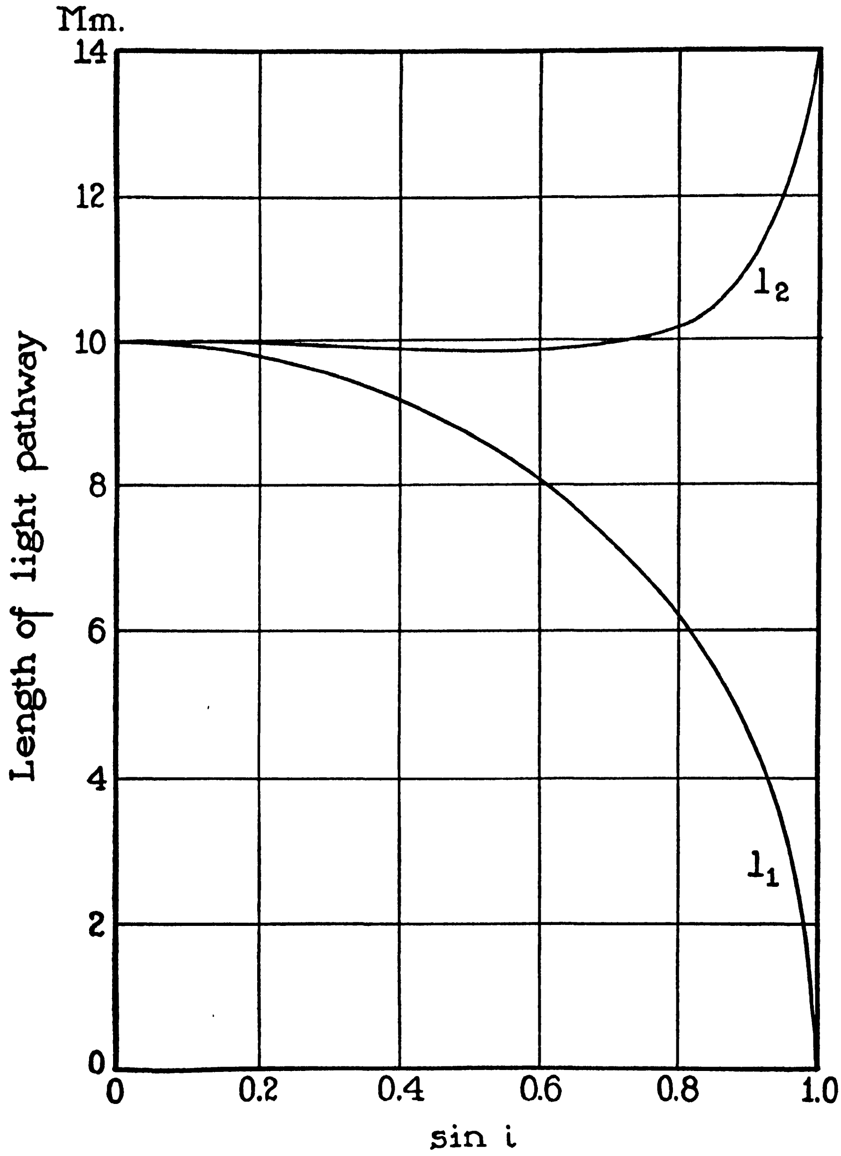

FIG. 2. Lengths of light pathways in the two quadrants of the cell shown in Fig. 1 *A* plotted against the sine of the angle of incidence of each ray on the cell. The areas under Curves l_1 and l_2 are proportional to the total absorptive paths within the front and back halves of the cell respectively.

III

As light passes through the cell, it is absorbed at a rate determined by the value of the absorption coefficient. In order that more light may be absorbed in the far half of the cell, the intensity of light reaching that half must not be too low. This means that in cells of the type which exhibit positive phototropism the absorption coefficient cannot exceed a certain critical value. The rest of this paper is given over to a solution for such critical values of absorption coefficient, and to a discussion of their meaning.[1]

Let I_0 = intensity of light incident on all points of the front of the cell
I_1 = intensity of any one ray at boundary between the front and back halves
I_2 = intensity of any one ray at the back wall
l_1 = length of any one light pathway in front half
l_2 = " " " " " " " back "
α = absorption coefficient of absorbing substance

Now for any one ray of light,

$$I_1 = I_0 \, e^{-\alpha l_1}$$

and

$$I_2 = I_1 \, e^{-\alpha l_2}$$

The light absorbed from this ray in the front half of the cell is therefore

$$I_0 - I_1 = I_0 \left(1 - e^{-\alpha l_1}\right)$$

and that absorbed in the back half is

$$I_1 - I_2 = I_0 \left(e^{-\alpha l_1} - e^{-\alpha(l_1 + l_2)}\right)$$

Since we are concerned with the *total* absorption of light in each half of the cell, the situation is not as simple as the foregoing treatment implies. In the first place, the loss of intensity by reflection at the front surface of the cell must be corrected for. Fresnel's formula was used to compute the loss of intensity by mirror-like reflection for each ray:

$$I_{\text{reflected}} = \tfrac{1}{2}\, a^2 \, \frac{\sin^2 (i - r)}{\sin^2 (i + r)} + \frac{\tan^2 (i - r)}{\tan^2 (i + r)}$$

---

[1] It is assumed that the effect of continuous light in producing phototropism is directly proportional to the intensity. The actual rate of the process which takes place in continuous light—presumably a sustained "softening" of the cell wall—and its relation to intensity are not known.

where

a = amplitude of incident light
i = angle of incidence at front of cell
r = angle of refraction at front of cell

The calculated percentage losses are given in Table I. They become large with values of i greater than 50–60°. They are probably slightly less than the real losses, since the refractive index of the cell wall is presumably higher than 1.38. Furthermore, a slight additional loss of intensity by *diffuse reflection* is neglected.

TABLE II

Calculation of absorption in front and back quadrants of the cell, for representative rays shown in Fig. 1 *A*. The cell radius is taken as 0.04 mm., α as 4. Detailed explanation in the text.

Angle of incidence	Sin i	l_1	l_2	Reflection loss	I_0	I_1	I_2	$I_0 - I_1$	$I_1 - I_2$
degrees		*mm.*	*mm.*	*per cent*					
0.0	0	0.0400	0.0400	2.6	0.974	0.831	0.710	0.143	0.121
11.7	0.203	0.0392	0.0400	2.5	0.975	0.833	0.712	0.142	0.121
23.8	0.404	0.0370	0.0397	2.6	0.974	0.840	0.718	0.134	0.122
37.0	0.602	0.0326	0.0394	2.9	0.971	0.850	0.726	0.121	0.124
44.5	0.701	0.0295	0.0396	3.3	0.967	0.859	0.733	0.108	0.126
53.4	0.803	0.0252	0.0404	4.6	0.954	0.863	0.737	0.091	0.126
64.3	0.901	0.0191	0.0418	9.6	0.904	0.838	0.710	0.066	0.128
72.3	0.953	0.0141	0.0460	18.3	0.817	0.772	0.643	0.035	0.129
76.0	0.970	0.0116	0.0506	26.0	0.740	0.707	0.578	0.033	0.129
90.0	1.0	0	0.0551	100.0	0	0	0	0	0

Knowing for each ray the value of I_0 corrected for loss by reflection, and assuming particular values of α, the next step is to calculate I_1 and I_2 separately for the rays shown in the diagram (Fig. 1 *A*), and to summate for the two quadrants of the cell the quantities of light absorbed. This procedure, while laborious, is direct, and unavoidable in the absence of a complete equation taking into account surface reflection and absorption within two halves of a cylindrical lens. Since l_1 is a function of the cell radius, more light will be absorbed in the front half of a large cell than of a small one. The following computations have been carried out for a cell of average size, the diameter being taken as 0.08 mm. Real values of l_1 and l_2 for this cell are obtained by dividing the corresponding, arbitrary lengths in Table I by 250.

Table II illustrates the calculations in a typical case when $\alpha = 4$. For the sake of simplicity, not all the light rays shown in Fig. 1 *A* are included. The intensity incident on all points of the front half of the cell is taken as 1. Column 6 gives the fraction of this intensity which enters the cell, obtained by subtracting the reflection loss for each angle of incidence from 1. Columns 9 and 10 give the amounts of light absorbed from each ray in the front and back quadrants of the cell respectively. These figures are plotted against sin *i*, and the areas under the two curves measured with a planimeter. The ratio of the two areas gives the ratio of light absorbed in the two quadrants, and therefore in the two halves, of the cell. In this particular case, when $\alpha = 4$

TABLE III

Ratio of light absorbed in the back half of the cell to light absorbed in the front half, as a function of the absorption coefficient. The cell radius is taken as 0.04 mm.

α	Absorption in back half / Absorption in front half
1	1.22
4	1.08
8	0.95
16	0.70

$$\frac{\text{Light absorbed in back half}}{\text{Light absorbed in front half}} = \frac{\int_0^1 (I_1 - I_2) d \sin i}{\int_0^1 (I_0 - I_1) d \sin i} = 1.08$$

Table III shows how this ratio varies with the absorption coefficient. Calculations similar to those illustrated in Table II were carried out for several values of α. With low values of α the ratio of light absorbed in the two halves is greater than unity, which means that there is greater photochemical action in the back half of the cell. As α increases, the ratio becomes progressively smaller, reaching 1 at a critical value of α which corresponds to equal absorption of light in the two halves of the cell.

Fig. 3 shows the ratio of light absorbed in the two halves of the cell plotted against α. The horizontal line through the ordinate 1 cuts the curve at the critical value of α for equal photochemical action in the two halves. With a cell of diameter 0.08 mm., this value of α is slightly greater than 6. As α approaches zero, the amounts of absorption become less and less, their ratio approaching the limiting value

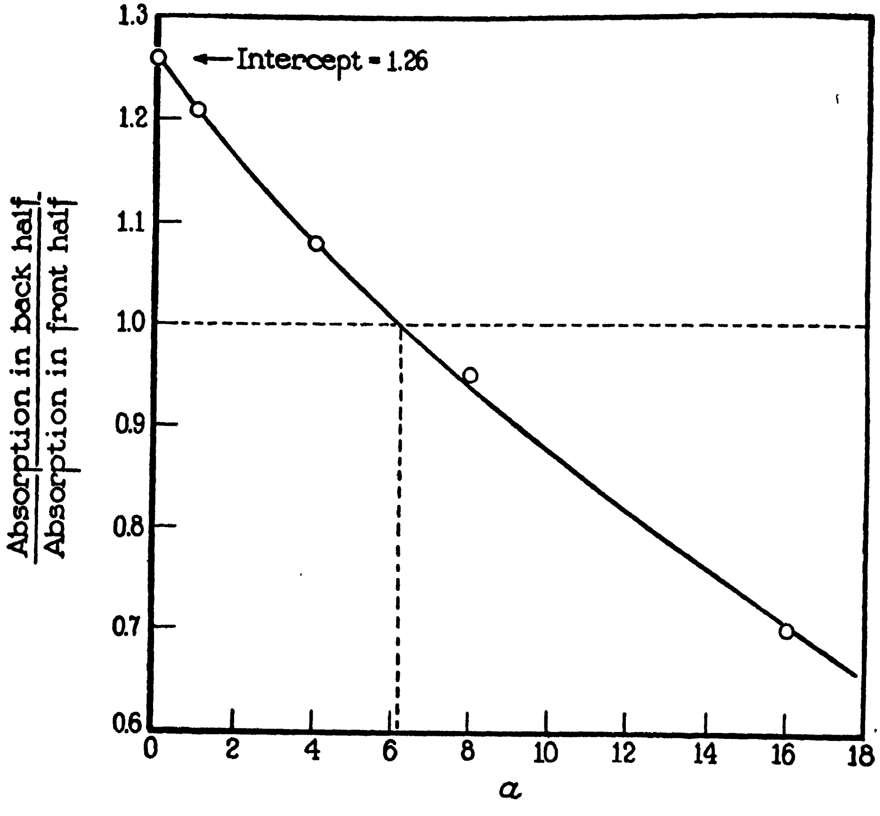

FIG. 3. Plot of the ratio of light absorbed in the back half of the cell to that absorbed in the front half, as determined by the absorption coefficient. The curve is for a typical cell having the radius 0.04 mm. For this cell, α must be less than about 6 if more light is to be absorbed in the back half. The ordinate intercept 1.26 is the greatest differential absorption possible for cells of refractive index 1.38 in air.

1.26. This is the ratio of the summated light paths in the two halves of the cell. It evidently represents the maximum differential absorption of light possible in a cylindrical cell of refractive index 1.38 illuminated with parallel light from one side in air. A cell of diameter 0.08 mm. must therefore possess a finite value of absorption coefficient somewhere between 0 and 6, in order that greater photochemical action may result in the far half.

For cells of different diameter, a family of curves similar to the one in Fig. 3 is obtained, all having the same ordinate intercept. Curves for cells of diameter less than 0.08 mm. have a smaller slope, and cut the dotted line through the ordinate 1 farther out on the abscissa. Curves for larger cells have a steeper slope and intercept the dotted line at values of α less than 6. The larger the cell, therefore, the smaller this critical value of α for equal photochemical action in the two halves of the cell. In the absence of direct measurements, it cannot be assumed that cells of all sizes contain the same concentration of pigment. If they did, the actual value of α in all cells would have to be less than the smallest critical value found for the largest cells, since all show positive phototropism.

Up to this point the partial reflection of light back into the sporangiophore at the back wall has been neglected. In Fig. 1 *B* the secondary rays reflected from the back wall of the upper quadrant of the cell are drawn. Fresnel's formula cannot be used to calculate rigorously for each ray incident on the back wall the intensity reflected back into the cell, in the way in which the reflection loss at the first interface was calculated, because the light which was originally refracted into the cell was polarized to a variable extent depending on the angles of incidence and refraction. Use of Fresnel's reflection formula as given above presupposes that the light is unpolarized.

A rough indication of the amount of light absorbed following partial reflection from the back wall may be obtained by neglecting this polarization effect, and by solving for the amount of absorption occurring in one typical, reflected ray.

Let I_0 = intensity of refracted ray entering the cell
I_2 = " " " " at back wall
I_3 = " " ray reflected from back wall
I_4 = " " reflected ray where it passes into the front half of the cell
I_5 = " " " " at front wall of the cell

l_1 = length of path of refracted ray in front half of the cell
l_2 = " " " " " " " back " " " "
l_3 = " " " " reflected " " " " " " "
l_4 = " " " " " " " front " " " "

Then additional light absorbed in the back half of the cell will be

$$I_3 - I_4 = I_3\left(1 - e^{-\alpha l_3}\right)$$

and additional absorption in the front half of the cell will be

$$I_4 - I_5 = I_3\left(e^{-\alpha l_3} - e^{-\alpha(l_3 + l_4)}\right)$$

Computations have been carried out for a typical ray incident on the front of the cell at an angle of 48.4°, where the cell radius was taken as 0.04 mm., α as 1, I_0 as 1, and the partial reflection from the back wall as 3 per cent. Under these circumstances the other quantities are as follows:

$l_1 = 0.0276$ mm.
$l_2 = 0.0399$ "
$l_3 = 0.0540$ "
$l_4 = 0.0165$ "

$I_2 = 0.9346$
$I_3 = 0.0281$
$I_4 = 0.0266$
$I_5 = 0.0261$

Amount of light absorbed prior to reflection $= I_0 - I_2 = 0.0654$
" " reflected light absorbed $= I_3 - I_5 = 0.002$
Reflected light absorbed in back half of cell $= I_3 - I_4 = 0.0015$
" " " " front " " " $= I_4 - I_5 = 0.0005$

In the case of this typical ray, three times as much reflected light is absorbed in the back half of the cell as in the front, if further reflections are neglected. Yet the reflected light which is absorbed is only 3 per cent of the absorption which takes place in the refracted ray prior to reflection.

The net effect of the reflected light is therefore to increase slightly the photochemical action in the back of the cell relative to that in the front. This action serves to lower slightly the critical values of absorption coefficient which have been derived.

IV

Since protoplasm is not optically homogeneous, it is evident that light passing through it undergoes some loss by scattering, even though a cell of *Phycomyces* in air converges much light to a sharp focus behind the cell (*cf.* Castle, 1933). Some of the scattered light is absorbed, and the effect of the scattering and secondary absorption is to increase the apparent value of the absorption coefficient of the intracellular pigment. As solved for in the previous section, α therefore represents the absorption coefficient of the pigment plus an undetermined factor for scattering.

It has been shown that if l is measured in millimeters, for a cell of diameter 0.08 mm. α must be less than 6, the approximate critical value for equal photochemical action in the two halves of the cell. It cannot be said on the basis of the present measurements how much less than this α is. Because of the small dimensions of the optical system of the cell, rather high values of absorption coefficient are compatible with the occurrence of positive phototropism.

Bending of a cell of the *Phycomyces* type is surely due to a difference in the extensibility of the wall on opposite sides of the cell. With high turgor pressure as a driving force, a slight difference in extensibility between opposite walls of the cell will lead to marked curvature. The mechanism outlined in this paper makes possible under the conditions described the absorption of light in one half of the cell up to an amount 1.26 times that in the other half. It is interesting to compare in this connection the observations of Massart (1888), who found that cells of *Phycomyces* placed between two sources of light opposed at 180° bent perceptibly toward the higher intensity when the ratio of intensities on opposite sides of the cells was 1:1.18.

It might still be objected that apart from photochemical considerations, the mere concentration of light in the far half of the cell shown in Fig. 1 *A* might somehow bring about a larger growth response than in the nearer half, by virtue of an anomalous sensitivity to the higher intensity. There is no evidence to support this idea. It is, in fact, shown incorrect by an ingenious experiment of Buder (1920). Sporangiophores placed in paraffine oil (refractive index = 1.47) exhibit negative phototropism, or bending away from a single source of light. Fig. 1 *C* is a diagram of the probable light paths within such a cell surrounded by a medium of higher refractive index. Under these circumstances the cell acts as a dispersing lens. No "concentration" of light occurs in the half of the cell nearest the light, yet a greater acceleration of growth takes place there. Buder's experiment therefore strongly supports the present view that the longer total path of light rays through the protoplasm of the far half of the cell is the significant factor in the phototropism of relatively transparent cells.

If the method of analysis used in developing the theory of differential absorption of light within two halves of a cell is correct, plane polarized light might be expected to be more effective in producing phototropic bending when polarized in the plane of incidence than at right angles to it. The amount of light entering the front of the cell should be greater when the vibrations of the incident rays are in the plane of the paper in Fig. 1 *A* than when the vibrations are perpendicular to that plane, although the energies of two such beams of light may be identical. Furthermore, the ratios of absorption in the back and front halves should be significantly different in the two cases.

SUMMARY

A physical basis is demonstrated, in the case of a cylindrical cell illuminated with parallel light from one side, for greater photochemical action in the half of the cell farthest from the source of light, when the cell is surrounded by a medium of refractive index less than that of the cell. Factors governing the balance and magnitude of unequal action of light in the two halves of the cell are: the refractive index of the cell, the cell radius, and the absorption coefficient of the intracellular pigment. A limiting value of absorption coefficient is deduced which cannot be exceeded in cells of a particular size showing positive phototropism. In terms of this mechanism the positive phototropism of *Phycomyces* in air is explained.

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THE ACTION OF THE PLANT GROWTH HORMONE

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INTRODUCTION

Although the control of cell elongation in plant tissues by a special growth-promoting substance or substances has been well established for some time, the processes by which this substance is able to bring about growth have remained obscure. Since the general properties of the response to growth substance by plant tissues, in particular of the *Avena* coleoptiles which have been most extensively studied, have been recently summarized by Thimann and Bonner (1933), only the principal points of interest for the present discussion need be given. These are briefly as follows:

(*a*) The growth-promoting substance of the *Avena* coleoptile is produced only in the coleoptile tip and passes from there downward (Went, 1928). After removal of the tip new growth substance is formed by the uppermost cells of the stump ("physiological regeneration," Dolk, 1926).

(*b*) The growth of the *Avena* coleoptile is for some time proportional to the amount of growth substance supplied to it (Thimann and Bonner, 1933).

(*c*) The growth substance which enters the plant and causes growth cannot be recovered; *i.e.*, is used up (Went, 1928).

(*d*) Growth substance is an unsaturated acid of empirical formula $C_{18}H_{32}O_5$ (Kögl, Haagen-Smit and Erxleben, 1933) and readily loses its growth-promoting activity by oxidation.

(*e*) The growth substance is a true hormone, *i.e.*, it acts in minute amounts and bears no direct stoichiometrical relationship to the number of molecules of soluble substance transformed during growth into, for example, cell walls. Thus one molecule of growth substance causes an amount of growth of the *Avena* coleoptile at 27°C. which requires the changing of 3×10^5 molecules of hexose to cellulose in cell walls (Thimann and Bonner, 1933).

The changes in the physical properties of coleoptiles under the influence of growth substance have been studied to some extent. Heyn (1931), and independently, Söding (1931, 1932) have shown that the plasticity, and also to a considerable extent the elasticity, of the coleoptile is increased after action of growth substance, and that this increase is independent of whether growth has occurred or not; *i.e.*, this action of growth substance is preliminary to active elongation. Heyn also found an increase in extensibility in coleoptiles which had been plasmolyzed after growth substance action, so that it is the physical properties of the cell wall, and not of the protoplasm, which are changed. The action of growth substance has now been further studied, and a few of the results will be described in the present paper. This study has been made easier by discovery of the fact that short sections of coleoptiles grow at a rapid rate if immersed in a growth substance solution of suitable concentration.

This method of using coleoptiles is convenient because, under proper conditions, a large amount of growth takes place in a relatively short time, and the "physiological regeneration" mentioned in (*a*) occurs slightly or not at all. It has the added advantage that the effect of known concentrations of growth substance upon the growth of younger and older portions of the same coleoptile may be examined independently.

Methods

Avena plants of the pure line "*Siegeshafer*" were used and were kindly supplied by Dr. Åkermann of Svalov. The plants were grown in sand in the dark at a temperature of 25°C. and a relative humidity of 85–90 per cent, and were used when 4 days old. Tips 3 to 5 mm. long were removed from the plants 2 hours before using. During this period the plants use up a large part of the growth substance already present in them and the production of growth substance by the stump does not commence. At the end of the 2 hours they were cut with a special cutter into sections in general 3.1 mm. long and immersed in the solution to be investigated.

The growth substance solutions, for which the author is indebted to Dr. K. V. Thimann, were prepared from large scale culture of the fungus *Rhizopus suinus* (Bonner, 1932; Thimann and Dolk, 1933). They were purified to an activity of the order of 2×10^{-6} mg. per plant unit.

Some of the growth measurements were made with a 12 power binocular equipped with an eyepiece micrometer. With this arrangement, the growth of free sections which were placed in small open dishes could be conveniently measured. For more accurate measurements, a horizontal microscope having an enlargement of 15 diameters and equipped with a stand mounted upon a micrometer screw was used. The coleoptile sections were placed upon thin glass rods of just the diameter of the interior of the coleoptile. These rods were in turn mounted in paraffin inside a rectangular vessel with glass sides through which the growth of the sections could be easily followed.

It should be mentioned here that considerable variability was found, in the reaction of sections to growth substance, both among plants of different experiments, and also to some extent among individual plants of the same experiment. A part of this variability is due, as will be shown, to slight differences in the ages of coleoptiles, but a considerable portion is probably due to causes not yet understood which also bring about variations in the standard *Avena* tests for growth substance activity from day to day. For this reason only the means of the results from a number of plants and several experiments can be regarded as significant.

EXPERIMENTAL

The growth of sections of coleoptiles, prepared in the manner already described, and immersed in pure water was first investigated. Table I gives the results of two series of measurements, and also a comparable series of measurements of sections with their bases in water, but their tips in air. It may be seen that the growth rate of the sections completely immersed in water falls steadily until after 7–8 hours it reaches a very low value. There is no sudden rise after 2 hours corresponding to a production of growth substance by the "physiological tip" as is the case with the sections whose tips were in air. The growth which took place in the sections immersed in water is to be attributed principally to growth substance in them when they were removed from the plants. Table II shows that the elongation of sections in growth substance solution is marked, being as great as 29 per cent in 4 hours and 55 per cent in 24 hours.

It was conceivable that with these sections immersed in solutions, the rate of diffusion of growth substance to the cut surface which it enters, or the rate of diffusion of oxygen to the tissue, might prevent a maximum growth response. Therefore measurements of the growth rates of sections both with and without stirring of the solution by air were made. These results are given in Table III, and show that stirring of the solution is not necessary.

The effect of the original position in the coleoptile of a given section upon its response to growth substance was then investigated. That such an effect exists is shown by Table IV, which gives the per cent growth per hour of sections from the tops and bottoms of a series of previously decapitated coleoptiles. Table IV is taken from one of seven experiments, all of which gave the same result. Similar measurements upon coleoptiles divided into more sections showed that the two 3.1 mm. sections nearest the apex of a plant decapitated 3–5 mm. from the tip have almost equal reactivity. The lower zones showed, as in Table IV, a lower ability to grow in the presence of growth substance. It has been known from measurements upon the growth rates of

TABLE I

Growth of Coleoptile Sections in Water

Experiment	Plants	Growth in per cent of original length per hr.						
		1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1 (tops in water)	6	1.8	1.5	1.2	0.7	0.6	0.4	0.4
2 (tops in water)	16	0.8	0.4	0.1	0.3	0.0	—	—
2 (tops in air)	6	2.5	2.5	4.0	4.3	4.7	3.3	—

TABLE II

Growth of Coleoptile Sections in Growth Substance Solution and in Water

(Each value is a mean from twelve–fifteen sections)

Solution	Growth in 2 hrs.	Growth in 4 hrs.	Growth in 24 hrs.
	per cent	*per cent*	*per cent*
Water	3	4	9
Water	3	5	7
Water	3	5	12
Growth substance	15	24	55
Growth substance	13	26	45
Growth substance	14	29	48

entire coleoptiles (marked into zones with ink or paper marks) that the lower zones do grow more slowly than those nearer the top. This has been attributed, however, to a lack of growth substance in the lower zones which must receive it through a long portion of coleoptile actively using the growth substance. That this is not the only factor is shown here directly, since it is clear that the cells at the base of the coleoptile show a much smaller growth response to growth substance than those nearer the top. In the present work, unless otherwise stated, only the two 3.1 mm. sections nearest the tip were used.

TABLE III

Growth Rates of Coleoptile Sections in Growth Substance Solution with and without Stirring by Air

Experiment	No. of sections	Growth per 2 hrs.					Total growth after 24 hrs.
		2 hrs.	4 hrs.	6 hrs.	8 hrs.	10 hrs.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1 (no air)	7	8	6	3	1	0.2	16
2 (no air)	6	9	4	3	2	0.3	19
3 (no air)	7	7	3	2	—	—	16
4 (air)	5	7	5	2	—	—	22
5 (air)	7	6	4	1	1	2	14
6 (air)	7	8	5	3	—	—	21
Mean of 1, 2, and 3	—	8.0	4.3	2.7	—	—	17.0
Mean of 4, 5, and 6	—	7.0	4.7	2.0	—	—	19.0

Growth substance concentration = 10 units per cc.

TABLE IV

Growth Rates of Top and Bottom Sections of Coleoptiles in Growth Substance Solution

	Growth per hr.						
	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Top sections	4.5	4.2	4.3	3.7	2.7	2.4	2.4
Bottom sections	1	1	1.1	1.4	2.1	0.9	0.9

Growth substance concentration = 10 units per cc.

The effect of concentration of the growth substance solution upon growth of the sections was determined, and is shown in Table V. The units are the standard growth substance units of this laboratory (Dolk and Thimann, 1932; Thimann and Bonner, 1933). From Table V it is evident that there is an optimum growth substance concentration in the region of 10 units per cc. Coleoptiles immersed in concentrations as great as 80 units per cc. show a shrinkage after 4 hours and at the end of 24 hours have frequently lost their turgidity due, apparently, to a toxic effect of the high concentration of growth substance. A decrease in growth in very low growth substance concentrations was also found. A simple consideration will show that only in the case of the 0.01 unit solution can this be due to an insufficient quantity of growth substance, and that even in the 0.1 unit solution the decrease must be due directly to the low concentration. From the data of Thimann and Bonner (1933) 0.00535 cc. of a 29 unit per cc. solution can give under their conditions a maximum of 7.85 mm. of coleoptile elongation. 1 cc. of a 0.1 unit per cc. solution could then give a maximum of 5.05 mm. total coleoptile elongation. Since in general in the present case twelve sections were placed in 4 cc. of solution, these sections should be able to elongate a maximum total of 22 mm., or 59 per cent per section, which is much larger than the 18 per cent observed. In the case of the 0.01 unit solution, however, each section should be able to elongate only

TABLE V

Effect of Growth Substance Concentration upon Growth of Coleoptile Sections

(Each value mean of 50–150 sections)

Growth substance concentration standard units	Growth in 2 hrs.	Growth in 4 hrs.	Growth in 24 hrs.
	per cent	*per cent*	*per cent*
80	3.3	2.1	0.4
40	4.3	8.0	7.2
20	7.4	10.8	15.4
10	11.7	19.9	31.0
1	8.4	15.7	27.0
0.1	6.5	12.1	17.5
0.01	4.5	7.0	15.5
0	3.3	5.6	11.9

5.9 per cent more than controls in pure water, and Table V shows that the increase is only 4 per cent.

Upon the theory that the action of growth substance is a simple physical change of cell wall, for example by decreasing directly in some way the viscosity of the substance in which cellulose micelles are imbedded (Heyn, 1931), one would hardly expect the action to be stopped by the presence of narcotics or cyanide. If, however, growth substance depends for its action upon processes of a metabolic nature, narcotics or cyanide should inhibit this action. It was easily demonstrated that both KCN and phenylurethane stop growth. Table VI gives a summary of two experiments with various concentrations of KCN and of phenylurethane and shows how marked is the stopping of growth. Further experiments showed that the concentration of KCN which brought about cessation of growth is 1–2 $\times 10^{-3}$ N, and that 0.05–0.1 per cent of phenylurethane brought about the same result. The concentrations of KCN are of the same order as those necessary to affect the respiration of other plant tissues (Schwabe, 1932).

TABLE VI

Inhibition of Growth of Coleoptile Sections by KCN and Phenylurethane

Solution	Growth	Solution	Growth
	per cent		*per cent*
Growth substance alone	23	Growth substance alone	20
Growth substance + 2×10^{-4} N KCN	5	Growth substance + 0.001 per cent phenylurethane	23
Growth substance + 10^{-3} N KCN	2	Growth substance + 0.01 per cent phenylurethane	14
Growth substance + 2×10^{-3} N KCN	−4	Growth substance + 0.1 per cent phenylurethane	2
Growth substance + 2×10^{-2} N KCN	−3	H_2O + 0.1 per cent phenylurethane	3
$H_2O + 2 \times 10^{-2}$ N KCN	−4		

That the growth resulting from growth substance does not occur in the presence of substances which stop metabolism suggests that the action of growth substance is itself intimately associated with the metabolism of the cell.

Experiments were then carried out to determine whether a connection between growth and cell oxidation exists. The action of growth substance in solutions under an atmosphere of pure nitrogen was first investigated. Commercial N_2 was passed over reduced copper in an electric furnace at 600°C. The gas was then cooled by passage through wash bottles, and bubbled through the solution containing the coleoptile sections. A preliminary experiment showed that growth substance is not affected in its activity by prolonged passage of N_2 through it. The sections were freed of O_2 by treatment with N_2 for 2 hours before introduction of the growth substance solution. After this preliminary 2 hours, sufficient growth substance was introduced to make the solution 10 units per cc., and the bubbling was continued for 4 hours more. The sections were then measured, a portion of them placed in growth substance solution in air, and the remainder in pure H_2O, in air. Table VII shows the results of five experiments. The coleoptiles were not harmed by the prolonged lack of O_2 as is shown by the fact that they grew normally upon being supplied with both growth substance and oxygen. However, a mean growth of only 3 per cent took place in N_2, although from Table V, 20 per cent growth would have taken place in air. That even this 3 per cent growth takes place is probably to be attributed to O_2 remaining in the sections. Therefore, normal growth fails to take place in N_2. Since the sections do not elongate, when placed in pure H_2O, to any greater extent than when immersed in H_2O without the pre-

TABLE VII

Inhibition of Growth of Coleoptile Sections by Nitrogen

Experiment	No. of sections	Growth after 4 hrs. in N_2 + growth substance	Growth after 20 hrs. in air + growth substance	Growth after 20 hrs. in air + H_2O
		per cent	*per cent*	*per cent*
1*	20	4.5	17	—
2	26	2.9	21	—
3	25	1.7	—	3.8
4	33	1.4	28	8.0
5	46	4.5	13	4.5

* In this experiment the sections were not first freed of oxygen.

liminary growth substance-N_2 treatment, it follows that either the action of growth substance has not taken place or else the growth substance has not been taken up by the sections.

The effect of the presence of KCN and of lack of oxygen in stopping growth suggested that a more intimate study of the effect of growth substance upon respiration be made. This was done with the aid of the standard Warburg manometers and using the technique described by Warburg (1926). The rate of respiration of the sections was found to be rather low and therefore it was necessary to use vessels having as small a gas space as possible in order to obtain measurable decreases in pressure due to O_2 uptake. These vessels did not contain alkali wells for absorption of CO_2, but depended for a decrease in pressure due to respiration, upon the greatly different solubilities of CO_2 and O_2 in the liquid present. The absolute amount of O_2 taken up in respiration may be calculated from the observed pressure change in the manner given by Gaffron (1929). This calculation requires a knowledge of the respiratory quotient. Since a preliminary determination showed that this quantity is close to unity for both sections in growth substance and sections in pure buffer, all of the subsequent calculations were based upon the assumption that the respiratory quotient was actually 1. This procedure is justifiable since the measurements are principally for purposes of comparison.

From 89 to 150 sections (including the basal portions of the stump) were placed in each vessel in M/50 phosphate buffer (pH = 4.8). The vessels were then attached to the manometers and placed upon the shaking rack, with the vessels immersed in a thermostat at 25°. These operations were carried out in red light in order not to cause any phototropic reactions in the sections. Some hours were needed for the respiration to reach a constant value after immersion in the vessel. This undoubtedly was due to an initial high rate of respiration of the wounded tissue at the cut surfaces. After a constant rate had been attained, sufficient growth substance to make up the desired concentration was introduced into one of a pair of vessels. The control vessel was either untreated, or, alternatively, a volume of water equal to the volume of growth substance solution used in the first vessel was introduced. Fig. 1 shows the course followed by the rate

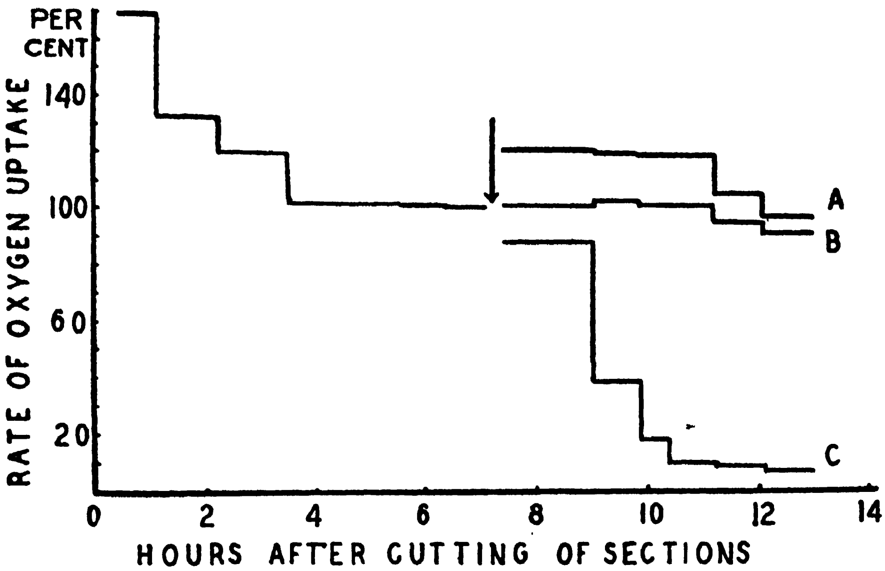

FIG. 1. Effect of growth substance upon the rate of oxygen uptake by coleoptile sections. At the arrow there were introduced: *A*, growth substance, 110 units per cc.; *B*, growth substance, 110 units per cc. inactivated; *C*, growth substance, 1100 units per cc.

TABLE VIII

Effect of Growth Substance upon Respiration of Coleoptile Sections
(Mean values of two to seven determinations)

Growth substance concentration	Increase in O_2 uptake over initial respiration rate, mm.^{3} per section per min.				Age of coleoptile
	During first 2 hrs.		During second 2 hrs.		
		Increase		Increase	
units per cc.		*per cent*		*per cent*	*days*
1100	−0.0022	−24	−0.0083	−90	4
110	+0.0018	19	+0.0014	15	4
11	+0.0025	27	+0.0024	26	4
1.1	+0.0013	14	+0.0017	18	4
0.11	±0.0000	0	+0.0003	3	4
110	+0.0021	31	+0.0012	21	5–6

of oxygen uptake in three typical experiments. It is clear that the addition of growth substance to a concentration of 11 units per cc., *i.e.* a concentration which causes growth (see Table V), causes a marked increase in oxygen uptake. A similar increase was found in every experiment in which a suitable concentration of growth substance was used.

The effect of growth substance concentration upon the stimulation of respiration was next investigated. Table VIII gives the summarized results for a wide range of concentrations. The maximum stimulation is caused under these conditions, in the neighborhood of 11 units per cc. Stimulation is also caused by concentrations ten times as great, although such concentrations are shown by Table V

TABLE IX

Effect of KCN and Phenylurethane on Respiration of Coleoptile Sections

Experiment	Solution	Respiration in mm.^{3} O_2 per min. per section
	Successive additions to buffer solution	
1	(Initial rate)	0.0079
	KCN, 5×10^{-4} N	0.0074
	KCN, 1×10^{-3} N	0.0003
	Growth substance, 110 units per cc.	0.0001
2	(Initial rate)	0.0061
	Growth substance, 110 units per cc.	0.0094
	KCN, 5×10^{-4} N	0.0099
	KCN, 1×10^{-3} N	0.0001
3	(Initial rate)	0.0069
	KCN, 8×10^{-4} N	0.0005
	Growth substance, 110 units per cc.	0.0003
4	(Initial rate)	0.0090
	KCN, 1×10^{-3} N	0.0005
	Growth substance, 110 units per cc.	0.0004
5	(Initial rate)	0.0069
	Urethane, 0.005 per cent	0.0073
	Urethane, 0.025 per cent	0.0088
	Growth substance, 110 units per cc.	0.0107
	Urethane, 0.05 per cent	0.0030
6	(Initial rate)	0.0094
	Urethane, 0.05 per cent	0.0012
	Growth substance, 110 units per cc.	0.0007

to inhibit growth. It should be remembered, however, that the number of sections per cubic centimeter of growth substance solution was in the respiratory vessels five times that in the vessels in which growth was measured. A still higher concentration of growth substance may be seen to be immediately toxic to the respiration. The 1.1 unit per cc. solution also stimulates respiration markedly; the 0.11 unit solution slightly, if at all.

Before further discussion of Table VIII, a few other results will be described. Fig. 1 includes the effects which were found to result from the addition of growth substance inactivated by oxidation with H_2O_2. It was then determined whether the respiration, and the addition "growth substance respiration" in particular, could be stopped by the concentrations of KCN and phenylurethane which stopped growth. That the respiration is so stopped is shown by Table IX. From this table it is also apparent that the additional growth substance respiration does not markedly differ in its sensitivity to the two inhibitors from the normal respiration, and this may be taken as evidence that the two are actually identical.

DISCUSSION

A simple calculation shows that the increase in respiration cannot be due to actual oxidation of the growth substance itself. Under the conditions of the experiment all of the growth substance could be completely removed by the observed increase in oxidation in 2–3 minutes. Therefore the effect of growth substance is due rather to some kind of stimulation.

In Table VIII it was shown that coleoptiles which were used for respiration measurements at the age of 5–6 days instead of the usual 4 days give an increase in respiration upon the addition of growth substance. Measurements upon these sections from "old" coleoptiles showed that they do not, however, elongate in the presence of growth substance. Therefore the increase in respiration upon the addition of growth substance is not due to the process of actual elongation. However, from Tables VI and VII it is clear that in order for elongation to occur, or probably even for growth substance action preliminary to elongation, the presence of aerobic metabolism is necessary.

There remain two possibilities, namely: (*a*) the stimulation of respiration by growth substance has nothing to do with its action in growth; and is a secondary phenomenon attending its presence or the presence of closely allied impurities in the cell, and (*b*) the stimulation of respiration by growth substance "prepares" the cell for elongation, which may then occur if other conditions are suitable. Schwabe (1932) has shown that various amino acids in minute quantities stimulate the respiration of *Elodea*, *Fontinalis*, and *Potamogeton*. These substances do not, as far as investigated, bring about growth in *Avena* coleoptiles. In favor of the second alternative, the following parallels between the increase in respiration upon the addition of growth substance and the growth of these coleoptile sections may be pointed out: (*a*) Low concentrations of growth substance cause growth; they also stimulate respiration. (*b*) High concentrations of growth substance inhibit growth; they also inhibit respiration. (*c*) The optimal concentrations for the two processes are similar. (*d*) Both growth and (by definition) aerobic respiration cease in the absence of oxygen, even in the presence of growth substance. (*e*) Both growth and respiration (as well as the excess "growth substance respiration") are stopped by the presence of KCN (10^{-3}N), or phenylurethane (0.05 per cent).

These parallels between the increase of respiration by growth substance and the effect of growth substance in promoting growth make it seem possible that the former is a necessary condition of the latter. That the increase of respiration can take place without accompanying growth, as it does in the old coleoptiles, does not exclude this possibility, since the old coleoptiles are stiffer and less plastic (Du Buy, 1932). It might be argued, for example, that although growth substance exerts in these old coleoptiles its general action, yet these sections are already too stiff, due to excessive thickening of the cell walls, to permit of extension by the turgor pressure.

A connection between growth substance activity and respiration is supported from another angle by the work of Van Ameijden (1917), who showed that phototropic and geotropic response do not take place in nitrogen.

The relation between the respiratory activity of growth substance and the increase of the plasticity of the cell walls observed by Heyn and Söding remains obscure, however. There are several conceivable mechanisms by which this result might be brought about and a detailed investigation of this question is now under way.

SUMMARY

1. Sections of *Avena* coleoptiles are found to show a considerable elongation when suspended in solutions of growth substance.
2. This elongation does not take place in the absence of O_2 and is inhibited by KCN and phenylurethane.
3. The rate of respiration of sections of coleoptiles is increased by the addition of growth substance in concentrations which cause growth. High concentrations of growth substance inhibit growth and also respiration.
4. The increase in respiration is inhibited by KCN and phenylurethane in the concentrations which inhibit normal respiration. These concentrations are the same as those which inhibit growth.
5. From 2, 3, and 4, it seems possible that the increase in respiration caused by growth substance may be an essential part of its action in growth.

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ELECTRICAL RESPONSES FROM LATERAL-LINE NERVES OF FISHES. III*

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I

The lateral-line nerve of the catfish *Ameiurus nebulosus* has been found to be normally in a state of continuous activity due to the repetitive discharge of impulses arising from the neuromasts (Hoagland, 1932–33*a*, *b*). Responses of the neuromasts to a variety of mechanical stimuli were found to take place against this background of "spontaneous" activity, no responses to mechanical stimulation occurring in the absence of the continuous activity. The response was found to be modified by mechanical stimulation of the neuromasts, including vibrations from tuning forks, and also by temperature; the frequency of the discharge of nerve impulses varies with the temperature of the receptors according to the Arrhenius equation, yielding a mean composite temperature characteristic of 5000 calories.

The structure of the lateral-line system facilitates investigation since the mechanoreceptors are linearly arranged in groups, forming a sort of extended ear. It has been possible to analyze quantitatively nerve impulses set up by various numbers of receptor groups. By cutting away most of the receptors, and by chilling the remaining ones, impulses have been recorded from single fibers of the lateral-line nerve (Hoagland, 1932–33*b*). The observations have been extended with brook trout, *Salvelinus fontinalis*, and rainbow trout, *Salmo irideus shasta* (*gairdneri*), both of which show vigorous spontaneous activity of the lateral-line system similar in many respects to that described for *Ameiurus*.

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II

The procedure consists in baring the lateral-line nerve a centimeter behind the head and dissecting it free for 1 or 2 cm. It is then tied, cut cephalad, and the freed length is drawn across silver-silver chloride electrodes connected to the recording system. The action potentials of the nerve are amplified and recorded by an iron armature oscillograph (Matthews, 1928) used in conjunction with a camera and a standing wave screen. A loud speaker makes the amplified action potentials audible.

The spontaneous activity of the lateral-line nerves of trout was found, in general, to be considerably more vigorous than that of catfish (*cf.* Hoagland, 1932–33*b*, Fig. 6*c*). This is probably due to the fact that more sensory units are active in the trout. There are usually over a hundred lateral-line pores posterior to the place of operation in trout. In the catfish there were seldom more than thirty-two. As was found in the case of the catfish, responsiveness of the neuromasts to mechanical stimuli is only manifest in preparations showing the spontaneous discharge. Five out of fifty-two lateral-line nerves of trout showed complete lack of all activity (*cf.* Hoagland, 1932–33*b*).

Responses to movements of the water bathing the fish, and to bending the trunk, were similar to those described for catfish. Ripples in the water, currents of water, stroking the flank, and bending the trunk all increase the discharge; but the effects on the photographs were partly obscured by the great vigor of the background of spontaneous activity. Tuning forks applied to the outside of the vessel containing the immersed fish appeared to increase the discharge in some cases during the time of application, but this response was never as clear as it sometimes is with catfish (*cf.* Hoagland, 1932–33*a*).

It was previously suggested that the receptors of the lateral-line responding to touch and movements of the trunk might be different from those producing the spontaneous activity. This suggestion was based on the fact that in the catfish it appeared that additional nerve fibers are brought into activity by these stimuli, giving, on the whole, larger action potentials than those repetitively firing in the absence of external stimulation (Hoagland, 1932–33*a*, Fig. 2). That a dual group of receptors exists is indicated by Fig. 1*a*. This shows impulses due to stroking the skin with a feather above the lateral-line

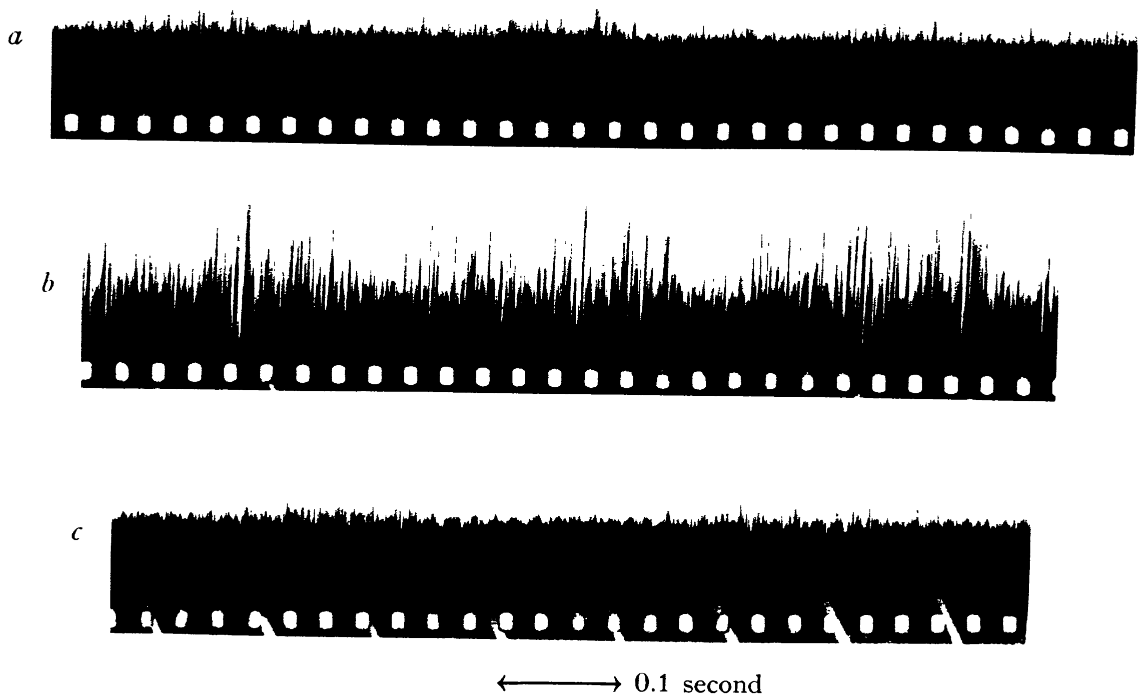

FIG. 1. (*a*) Three bursts of impulses from a lateral-line nerve of a catfish in response to stroking the skin over the lateral-line canal. Exactly the same effect is produced by concave bending of the trunk on the tested side. The bursts are recorded against a diminished spontaneous discharge from only two groups of neuromasts.

(*b*) Similar responses in a trout. Here some twenty-eight possible neuromast groups remained and contributed to the spontaneous discharge.

(*c*) Same as (*b*) except at 1·5 the amplification. Two bursts are shown. The figure illustrates the fact that fibers giving larger action potentials than those mediating the spontaneous discharge are brought into action by the stimulation.

canal, after slicing through the lateral-line system in a catfish posterior to the region of exit of the nerve, in such a way as to leave only one active group of neuromasts. With the single neuromast group giving only sporadic impulses the additional impulses set up by the feather strokes clearly occur in nerve fibers, as judged by the all-or-nothing height of the potential waves, which are different from those spontaneously discharging.

In catfish the tactile receptors, as judged by their responses to touch, are located along the entire length of the canal with their maximum distribution in the anterior portion of the trunk. In trout the maximum distribution of tactile receptors is found in the posterior part of the lateral-line canal within several centimeters of the tail. Stroking this region of the trunk above the canal produces vigorous bursts of impulses against the background of the spontaneous discharge. Few impulses are produced in trout by stroking the flank at a level with, or cephalad to, the dorsal fin. If one hangs a dead trout tail down and bends the body from the tail, the place of maximum bending occurs at a position coinciding exactly with the region of maximum distribution of tactile receptors found by means of the electrical recording technique.

The distribution of special pressure receptors along the lateral-line canals of trout and catfish is interesting in the light of the general habits of the two forms. Catfish are sluggish animals showing distinct negative phototropism and feeding along the bottom mostly at night. The region of maximum distribution of the pressure receptors of the lateral-line canal occurs well forward in the trunk in a position where the receptors might be stimulated by contact with objects in the course of the animal's progression. Reflex swimming movements excite these receptors (Hoagland, 1932–33*a*), presumably by pressure from surrounding tissues. In this way they may serve as proprioceptors. In trout, which are active and vigorous swimmers and which are not conspicuously negatively phototropic, the pressure receptors are located in a position near the tail where direct tactile stimulation by objects in the water would be very unlikely to occur. Their position for proprioception is, however, ideal. The remarkable control of trout over their swimming movements may, in part, depend on this more efficient distribution of pressure receptors responding to trunk flexion. The threshold of excitation to flexing as indicated by the extent of bending necessary to produce impulses, is lower in trout than in catfish. Figs. 1*b* and 1*c* show responses of trout pressure receptors to stroking the skin over the lateral-line canal. Exactly the same effects are produced by concave flexing of the trunk. As judged by the density of nerve impulse discharge recorded by the loud speaker when the skin is explored by a pointed instrument, the cutaneous region directly over the pores of the trout lateral-line shows maximum sensitivity. In catfish the pores seemed no more sensitive than the region of skin between them. If one fills a medicine dropper with water and holds the submerged tip several millimeters above an immersed lateral-line pore, in the region of maximum distribution of tactile receptors in trout, one obtains nerve impulses in response to extremely light compression of the bulb of the dropper. Very slight downward movement of the column of water stimulates. The threshold for the fish receptors is considerably lower than that for pressure receptors in the human finger tip, since under similar conditions the bulb of the dropper must be pressed considerably more in order to excite tactile sensations from immersed finger tips.

In the experiments made in obtaining Figs. 1*b* and 1*c* the lateral-line nerve was drawn across the electrodes just anterior to the region of maximum distribution of tactile receptors near the tail. By taking the nerve from the flank to the electrodes from the posterior region of the trunk just anterior to the region of maximum distribution of tactile receptors, one gets a preparation in which there remain only some 20 (instead of 100) neuromast groups supplied by the nerve and contributing to the spontaneous activity. If one now slices through the lateral-line system a centimeter caudal to the region of exit of the nerve, a preparation is obtained in which only a few neuromasts are spontaneously active. Impulses initiated by stroking or flexing the body stand out clearly against the background of reduced activity (Figs. 1*b*, 1*c*); these occur in fibers not normally concerned in the spontaneous activity, since the potentials of the individual fibers set up in response to the stimulus are clearly larger on the average than are those of the spontaneous discharge.

In both trout and catfish impulses are set up only on the side in which compression of the receptors occurs; *i.e.*, the side of concave bending.

Straightening and convex bending of the side do not produce impulses. The threshold for the effect of bending is lower in the trout than in the catfish. Adaptation of the response to bending or direct pressure is very rapid; impulses other than the spontaneous discharge are set up only in response to changes in the stimulus. In swimming, receptors on the two sides are discharged alternately corresponding to bendings of the flank, at frequencies depending upon the speed of movement. These impulses may serve a useful proprioceptive function despite the fact that experiments in which lateral-line nerves have been cut do not as a rule show marked disturbances of normal swimming movements. Disequilibration reported from time to time by different investigators resulting from lateral-line nerve cutting may be due to interference with proprioceptive impulses, although my own observations indicate that little if any disequilibration in normal swimming results from cutting the lateral-line nerves. It is more probable that the proprioceptive impulses may serve a tonic, reinforcing function for vigorous swimming, since the amount of the discharge increases both with the extent of flexion and with the rate of flexion.

SUMMARY

Evidence indicates that lateral-line fibers, other than those mediating the "spontaneous" activity of the lateral-line receptors, are brought into play in response to pressure stimuli in catfish and in trout.

The distribution and mode of stimulation of mechanoreceptors along the lateral-lines of trout and catfish are discussed in relation to the natural activities of these forms.

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SOME METHODICAL ERRORS WHICH MAY ARISE IN THE DETERMINATION OF BOUND WATER*

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"Bound water" has been a subject of unusual interest to the physiologist (1) in recent years and many papers of merit have appeared upon the question.

These methods and concepts of the physiologist have been attended by a parallel development in the realm of pure physical chemistry. Thus Polanyi *et al.* (2) have developed the so called potential theory of adsorption and have studied the decrease in the adsorption potential as the distance from the particle is increased. This adsorption potential is calculated by means of the equation

$$A = RT \ln \frac{P_s}{P}$$

in which P_s is the vapor pressure of the liquid at the given temperature and P is the vapor pressure of the material in equilibrium with the adsorbed material. It is the adsorption potential which determines the extent to which the vapor pressure of a gas is to be reduced upon approaching a solid surface. On the immediate surface the potential is exceedingly high, the vapor pressure and the fugacity of the gas are low. Assuming for the moment that we are dealing with some liquid below its critical temperature, perhaps water, we can then say that its activity has been correspondingly reduced. As we increase the amount of water vapor present we eventually reach the saturation point. It is hard to see how the addition of further water could change the situation. In other words, if the substance with its adsorbed water were immersed in water the condition at the surface would not be changed. We should have under these conditions what the physiologist has been calling "bound water." Its activity has been decreased and its ability to dissolve substances has been correspondingly diminished. This, it seems to the author, is the physical picture of bound water.

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If, however, we add to this system a small amount of some solute—perhaps sucrose—we upset the conditions. This same potential which contributed to the diminution of the vapor pressure and activity of the water now diminishes the activity of the sucrose which comes in the proximity of the solid surface. The sucrose has been adsorbed. The author is aware that Polanyi has endeavored to apply his adsorption theory to solutions with but small quantitative success. However, the author does feel that the theory is a sound one and that the lack of quantitative agreement is due to the extremely complicated situation. In any event, however, that the solute can be adsorbed by colloidal material has been demonstrated a great many times and is a fact, whatever may be the theory by which we account for the phenomenon.

It appears to the author that adsorption of the solute by the substrate may be a source of large errors and it may turn out that this is the principal cause of disagreement so generally noticed between the results of different workers. It is true that it has been recognized that adsorption might play a rôle, but there seems to be a lack of appreciation of size of errors which might result from a relatively small adsorption of the solute.

In a recent paper Greenberg and Greenberg (3) presented results obtained from a study of the concentration of the solute in the ultrafiltrate from certain colloidal materials. This concentration was compared with the concentration of the solute in the original colloid. They proposed the following equation

$$h = \frac{1}{P}\left(1 - \frac{C_T}{C_u}\right)$$

where h is the bound water per gram of colloid, P is the amount of the colloid per gram of total water, C_T is the concentration of the reference substance per gram of total water in the system, C_u is the concentration per gram of water of the reference substance in the ultrafiltrate. They presented data from which the bound water might be calculated by the use of this equation. The author has used their

TABLE I

	Gelatin concentration	Solvent composition	Reference substance	(C_T)	(C_u)	Bound water h	C_T corrected	Bound water corrected
1	0.0165	0.01 N HCl	Urea	0.00109	0.00109	0.0	0.00104	3.03
2	0.0220	0.025 N HCl	"	0.00200	0.00205	1.109	0.00190	3.327
3	0.0267	0.10 N HCl	"	0.00129	0.00120	−2.808	0.001226	−0.798
4	0.0254	0.075 N HCl	"	0.00140	0.00125	−4.72	0.00133	−2.52
5	0.0274	0.26 N HCl	"	0.00138	0.00138	0.0	0.00138	1.825
6	0.0300	0.011 N NaOH	"	0.00099	0.00095	−1.40	0.0094	0.35
7	0.0370	0.005 N KCl	"	0.00113	0.001138	0.19	0.001074	1.62
8	0.0290	0.01 N NaCl	"	0.00211	0.00205	−1.008	0.002005	0.8413
9	0.0273	0.0075 N KCl	"	0.00157	0.00160	0.6886	0.001492	2.48
10	0.0300	H_2O	"	0.001415	0.00139	−0.599	0.001344	1.10
11	0.0300	H_2O	Glucose	0.001	0.00099	−0.337	0.00095	1.35
12	0.0338	H_2O	"	0.001	0.001008	0.235	0.00095	1.701
13	0.0311	H_2O	"	0.001	0.00100	0.0	0.00095	1.61
14	0.0263	0.1 N NaCl	"	0.001	0.000994	−0.228	0.00095	1.68
15	0.0316	0.0043 N HCl	"	0.001158	0.001159	0.275	0.00110	1.61
16	0.0500	H_2O	Urea	0.00987	0.00980	−0.143	0.00937	0.816
17	0.0400	0.005 N NaOH	"	0.00987	0.00984	−0.076	0.00937	1.118
18	0.0400	0.091 N HCl 0.08 N NaCl	"	0.02670	0.02660	−0.0938	0.00254	1.127
19	0.0500	0.1 N KCl	"	0.02670	0.02680	0.0761	0.00254	1.044

TABLE II

	Casein concentration	Solvent composition	Reference substance	C_T	C_u	Bound water h	C_T corrected	Bound water corrected
(*a*)	0.0325	0.023 NaOH	Urea	0.001435	0.001425	−0.216	0.00136	1.34
(*b*)	0.0228	0.05 KOH + 0.01 KSCN	"	0.00145	0.00145	0.0	0.00137	2.21
(*c*)	0.0175	0.01 KOH + 0.02 $KC_2H_3O_2$	"	0.00078	0.00079	0.725	0.000741	3.55
(*d*)	0.0118	0.0067 KOH + 0.03 $KC_2H_3O_2$	"	0.00078	0.00078	0.0	0.000741	4.24
(*e*)	0.0346	0.030 KOH + 0.019 $KC_2H_3O_2$	"	0.00061	0.00060	−0.479	0.00058	1.012
(*f*)	0.0144	0.012 KOH + 0.0145 $K_2C_2O_4$	"	0.00057	0.00059	2.35	0.00054	5.77
(*g*)	0.0266	0.0145 NaOH	Glucose	0.000979	0.000986	0.269	0.00093	2.135
(*h*)	0.0294	0.0154 NaOH	"	0.000882	0.000882	0.0	0.000838	1.697
(*i*)	0.0227	0.0119 NaOH	"	0.000909	0.000907	−0.0969	0.000864	2.088

data and their equation for bound water and has calculated the amount of water bound or associated with each gram of colloid. This has been done both for gelatin and casein. The results of these calculations are presented in Tables I and II.

The results can be seen to be extremely erratic. In some cases a tremendous amount of *negative* bound water is found (Table I, 0.0254 per cent gelatin, 0.075 N HCl, and 0.00140 gm./cc. urea). In five cases the amount of bound water is zero. In others a positive value is found. From these erratic results we can judge that the method is capable of only a small degree of accuracy. Assuming, however, for the moment that the experimental results describe completely the actual conditions, we make the further assumption that 5 per cent of the solute is adsorbed, *i.e.*, bound by the substrate—surely a modest estimate. We have then recalculated the data with the results as shown in Column 9 of Table I and Table II.

We have used in these calculations the modified formula

$$h = \frac{1}{P}\left(1 - \frac{C_T - S}{C_u}\right)$$

where S is now the amount of the solute adsorbed per gram of total water.

It can be seen that this small correction changes the final values in some cases by over 300 per cent and in many cases the mysterious *negative* bound water becomes a more reasonable positive value. This represents a serious and drastic revision of the results and conclusions of Greenberg and Greenberg.

SUMMARY

The "bound water" hypothesis has an adequate theoretical basis. A relatively slight adsorption of the solute along with water molecules (bound water) will explain the failure of *certain technics* to demonstrate the existence of bound water in biochemical systems.

I wish to thank Dr. R. A. Gortner for his helpful criticism of this paper.

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1. Gortner, R. A., in Luck, J. M., Annual review of biochemistry, Stanford University Press, 1932, **1**, 21.
2. Polanyi, M., *et al.*, *Ber. deutsch. phys. Ges.*, 1914, **16**, 1012; 1916, **18**, 55; *Z. Elektrochem.*, 1920, **26**, 370; *Z. phys. Chem.*, 1928, **132**, 384.
3. Greenberg, D. M., and Greenberg, M. M., *J. Gen. Physiol.*, 1933, **16**, 559.

ANESTHESIA PRODUCED BY DISTILLED WATER

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Experiments on *Nitella*[1] show that the cells lose their irritability when transferred from pond water or from a nutrient solution to distilled water. The process appears to be perfectly reversible as the normal irritability returns when the cells are replaced in the nutrient solution. Hence anesthesia appears to be produced by removing something from the cell.

The recording apparatus is essentially an electrostatic short-period voltmeter,[2] consisting of a Cambridge Type *A* string galvanometer with thermionic amplifier. The circuit arrangement is shown in Fig. 1. A selected 201 *A* vacuum tube with a grid to cathode D.C. resistance of over 100 megohms at free-grid potential is employed. This is used at free-grid potential without a grid leak, under which conditions the error in measurement of potential of a *Nitella* cell is less than 1 per cent. By the use of a series calibration (P_1) the error is reduced to a negligible value.

In operation, the plate-circuit resistance is adjusted until equal to the internal resistance (plate-cathode) of the tube at free grid.

The grid-biasing potentiometer (P_2) is then adjusted to free-grid potential. The *Nitella* cell or a calibration potential may now be thrown into the grid circuit, and the sensitivity of the instrument brought to the required value by use of the galvanometer shunt R_1 or by changing the tension of the string.

By operating at free-grid potential the grid current is kept at a minimum value and the galvanometer is protected if the circuit is accidentally opened. The use of plate-circuit resistance equal to the internal resistance of the tube gives maximum power amplification. Ample power is secured to operate the string galvanometer while drawing minimum current from the cell under examination.

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[1] This is *Nitella flexilis* Ag., the species used in all previous work from this laboratory.

[2] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, **11**, 391. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929–30, **13**, 547; 1930–31, **14**, 385, 473. Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 361.

Reference to Fig. 2 will show the reason for employment of the amplifier. The voltage existing in the *Nitella* cell is distributed across R_1 and R_2 in direct proportion to their resistance. Four times the voltage applied to the grid circuit (R_2) of the 201 *A* tube is generated in the plate-circuit (R_3) which has only about 1/40 the resistance of the *Nitella* cell. The sensitivity of the galvanometer is about 160 times as great as it would be without the amplifier, while the current drawn from the *Nitella* cell is less than 1/100,000 of that which would be drawn by the galvanometer if connected direct to the *Nitella* cell.

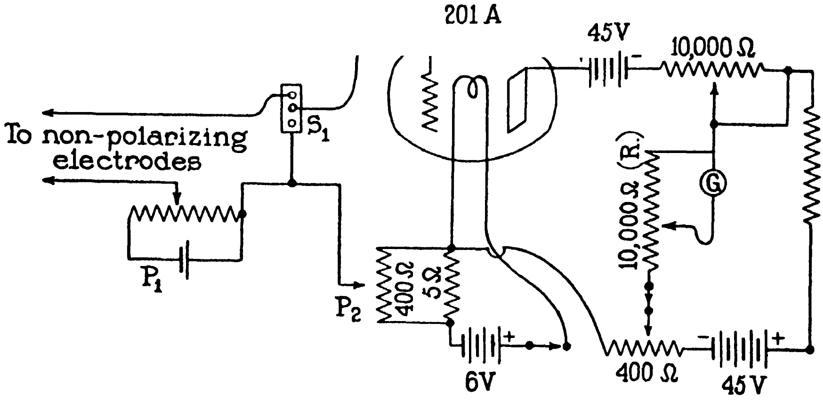

Fig. 1. Thermionic amplifier for galvanometer

G = Cambridge Type *A* string galvanometer

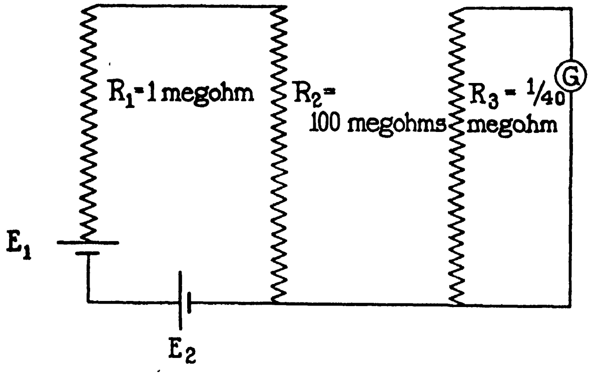

Fig. 2. Schematic diagram of vacuum tube amplifier employing 201 *A* radiotron.

E_1R_1 = voltage and resistance of *Nitella* cell.

E_2 = calibrating E.M.F. from source of negligible resistance (50 ohms per volt).

R_2 = grid to cathode resistance of 201 *A* radiotron.

R_3 = plate to cathode resistance of 201 *A* radiotron.

The temperature in all experiments was 21–23°C. The experiments with distilled water were carried out in the following ways:

1. Mass Cultures

Cells were placed in a covered pan of enamelled ware containing a mixture of electrolytes which will be called Solution *A*. These gave action currents for 2 weeks[3] during which period they were tested frequently. Solution *A* was then replaced by distilled water and left until their irritability was lost. When these cells were replaced in Solution *A* most of them regained their former irritability in 2 days: in some of these which were kept in distilled water for 8 days anesthesia doubtless lasted a week since irritability probably disappeared in a day.

"Loss of irritability" as used in this paper means loss of ability to respond to a definite stimulus which was approximately the highest that could be applied without danger of injury (see p. 93). This was direct current applied for 3 seconds by means of two silver-silver chloride electrodes placed (about 1 cm. apart) at one end of the cell. A cell which does not respond in this length of time will not do so when the stimulus is prolonged indefinitely.

Cells were regarded as showing normal irritability when they responded to 50 to 160 mv. applied in this manner. In this experiment cells which did not respond to 400 mv. were regarded as having lost their irritability.

In a typical experiment, such as is described above, 25 cells were kept in Solution *A* for 2 weeks and all gave action currents (with 160 mv.) before being placed in distilled water. But of the 23 cells alive after 1 day in distilled water only 48 per cent responded and of the 21 alive after 3 days only 9 per cent responded. Replaced in Solution *A* 50 per cent responded after 1 day, 70 per cent after 2 days, 77 per cent after 3 days, 77 per cent after 4 days, and 87 per cent after 10 days. (During the experiment 68 per cent of the cells died: no account is taken of these in making up the percentages, which refer only to the living cells.) There was no loss of irritability during 16 days in the control cells kept in Solution *A*, except in dead cells (46 per cent of these cells died in 10 days and 53 per cent in 16 days).

The cells, placed on paraffin blocks, were surrounded by moist air except where the contacts, consisting of moist cotton, were applied. The contacts were about 1 cm. apart.

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[3] Cells often remained normal under these conditions for 10 weeks or more (each was a single cell from which neighboring cells had been cut away).

The water was redistilled, using a pyrex glass flask and condenser, and rejecting the first third of the distillate (baffle plates were used to prevent mechanical contamination).

Solution *A* contains

$CaCl_2$	0.001 M	Citrate	0.00001 M
NH_4Cl	0.00025 M	Tartrate	0.00002 M
$MgCl_2$	0.00025 M	Phosphate	0.00003 M
$NaHCO_3$	0.001 M		

To 1000 parts of this 1 part of sea water was added.

2. *Individual Test-Tubes*

In order to follow the behavior of individual cells 40 test-tubes were filled with Solution *A* and a cell was placed in each. Each of these cells gave action currents when stimulated electrically (160 mv.). Solution *A* was replaced by distilled water and the cells were again tested 24 hours later, at which time all responded.

Let us now consider the behavior of a typical cell (No. 3). After 2 days in distilled water it still gave action currents but after 3 days it no longer responded when 300 mv. were applied and it was therefore regarded as having lost its irritability. It was then transferred to Solution *A* and 24 hours later gave action currents: this was also true 2 days later when the experiment was discontinued.

We see that ability to respond to 300 mv. disappeared in about 3 days in distilled water and was regained in Solution *A* in about a day. Control cells, kept in Solution *A*, showed no loss of irritability (except in the case of those that died during the experiment).

Leaving out of account 23 cells which died[4] during the experiment, let us consider the 17 which survived to the end (7 days). All were treated like Cell 3. Of these, 7 behaved like Cell 3 and the rest differed only in minor details (with the exception of 2 which continued to give action currents throughout the entire experiment). For example, 4 lost their irritability more quickly than Cell 3 at the start (*i.e.* after 1 day in distilled water) but all save 1 regained their irritability just as quickly as Cell 3 when transferred (on the 5th day of the experiment) from distilled water to Solution *A*. When this transfer was made all the cells except 4 had lost their irritability but all save[5] 2 regained it in 24 hours in Solution *A*. All the cells responded when the next test was made 2 days later (8th day of the experiment).

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[4] The mortality seems to be due principally to the handling of the cells which have to be removed from the test-tubes for each test. Cells kept continuously in Solution *A* die much more quickly when handled than otherwise.

In order to see whether the restored irritability could again be removed another experiment was made with a lot of 40 cells. A typical cell (No. 7) lost its irritability after 1 day in distilled water (no response to 500 mv.): tests on the 2 following days showed that this condition persisted. It was then transferred to Solution *A* where it regained its irritability in 1 day: a test on the following day showed that it was still responsive. It was then transferred to distilled water and a test 2 days later showed that it had again lost its irritability.

Hence it is possible to remove irritability, restore it, and take it away again.

Leaving out of consideration the 29 cells which died during the experiment, we may say that the 11 which survived to the end of the experiment (8 days) agreed with Cell 7 except in minor details. Thus at the start 6 were slower than Cell 7 in losing irritability (4 required 2 days and 2 required 3 days in distilled water). When the cells were afterward transferred to Solution *A* (after being 3 days in distilled water) 2 were slower than Cell 7 in regaining irritability: nevertheless they accomplished it in 2 days. When on the 6th day, after being 2 days in Solution *A*, the cells were transferred from Solution *A* to distilled water, 5 cells were slower than Cell 7 in losing their irritability but (with one exception) all these lost it in 3 days.

3. Treatment of Restricted Areas of the Cell

Under the circumstances it seemed worth while to ascertain whether distilled water applied to a restricted area of the cell would cause a local loss of irritability. This proved to be possible and it was found that the irritability could subsequently be restored.

A typical cell (No. 3) behaved as follows: With Solution *A* at *A*, *B*, *C*, and *F* (Fig. 3 with *D* and *E* omitted) stimulation (160 mv.) by means of the electrodes at *A* and *B* resulted in responses at *C* and *F*. The solution at *F* was then replaced by distilled water. After 24 hours *F* did not respond to 300 mv. but *C* gave a normal response. Tests on each of the 8 following days showed this situation to be unchanged.

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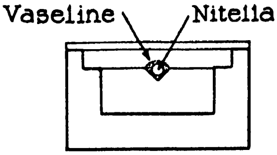
[5] These 2 regained their irritability later on.

The distilled water at *F* was then replaced by Solution *A* and after 24 hours *F* responded as well as *C*. In this case therefore local anesthesia lasted 8 days but there was no irreversible injury.



Section at XY

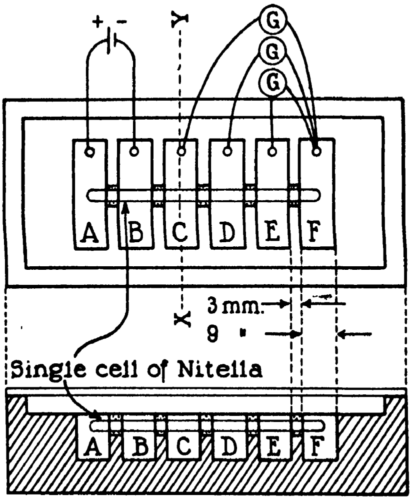


FIG. 3. A paraffin block containing 6 cups (*A*, *B*, *C*, *D*, *E*, and *F*) filled with solution is shown in cross-section above, in ground plan in the middle, and in longitudinal section below. Each partition has a notch filled with vaseline in which a *Nitella* cell is embedded as shown in the cross-section (above). In this way 6 different areas of the cell can be treated with different solutions. A cell may remain several days in the block without apparent injury.

Electrical connections are usually made as shown in the ground plan, *i.e.* from *C* to *F*, *D* to *F*, and *E* to *F* (here *G* signifies a string galvanometer with a vacuum tube amplifier; the apparatus is essentially electrostatic in principle).

In view of the fact that the protoplasm and sap of *Nitella* are in constant circulation it might be supposed that substances leached out of *D* by distilled water might be replaced from neighboring regions rapidly enough to preserve the irritability but this was not the case.

In this case 20 cells were employed. Leaving aside the 8 cells which died during the experiment we may say that the 12 which survived to the end of the experiment (11 days) behaved like Cell 3 except for minor differences. For example, at the start 3 were slower than Cell 3 in losing irritability at *F* but all lost it in 4 days. Furthermore 3 cells regained irritability at *F* somewhere between the 6th and 8th days although apparently in contact with distilled water (whether there was some contamination of the distilled water from the adjoining cup is not known). Also 5 cells took 2 days to regain irritability at *F* when (on the 8th day of the experiment) distilled water at *F* was replaced by Solution *A*.

The cells were placed in cups in paraffin blocks as shown in Fig. 3. These were prepared by running paraffin into steel[6] molds so as to make 6 cups separated by 5 solid partition walls of paraffin about 3 mm. in thickness. In the center of each of these a vertical notch was cut to admit a *Nitella* cell. When the cell was placed in the block, as shown in Fig. 3, the cups *A*, *B*, *C*, *D*, *E*, and *F* were filled with solution. No liquid crept from one cup into the next under these circumstances because the space in the notch around the *Nitella* cell was filled with vaseline. The block was covered with a glass plate.

There was, of course, a capillary film of liquid surrounding the cell wall but this did not produce more short-circuiting than the ordinary experiments in air or in a moist chamber such as have been described in previous papers.

It is evident that the area at *F* which is treated with distilled water gives no response when a stimulus is applied by means of an outgoing electrical current at *B*, but we are unable to say what would happen if a stimulus were to be applied directly to the region in contact with distilled water.

In order to answer this question two methods were employed both of which showed that no normal response could be obtained.

1. Cells were used which had been kept in a paraffin block (Fig. 3) with distilled water at *A*, *B*, *C*, *D*, *E*, and *F* for 3 days. These places remained in contact with distilled water while the following test was made. In the circuit through *A* and *B* 500 mv. were applied in the usual way with an ingoing current at *A* and an outgoing current at *B*. As this produced no normal response it was gradually increased to 1200 mv. We led off in the usual way from *C* to *F* with electrodes in both cups, but the partition between *B* and *C* was removed so that these two cups were in contact with the same area of the cell. The record showed an increased negativity at *C* due to the applied E.M.F. which ceased when the current was broken. However, the negative potential at *B* fluctuated irregularly during the outward flow in a manner suggesting a breakdown of the protoplasm by the large current employed and a consequent change in effective resistance. There was no transmission to

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[6] Steel was preferred to copper or brass since it is not toxic.

D, *E*, or *F*: all points were in contact with distilled water during the experiment. With 300 mv. applied there was no such fluctuation in the negativity of *C* during the flow.

2. In the second method the circuit from *A* to *B* formed one arm of an equal-arm Wheatstone bridge as described by Blinks.[7] The arrangement is shown in Fig. 4. We could then lead off to the galvanometer as shown in the figure and apply an electrical stimulus without having it affect the photographic record directly. In the record we see only the changes in P.D. which take place in the protoplasm. We found no normal responses at *B*: some irregular disturbances occurred at higher voltages but in no case were these followed by action currents at *C*, *D*, or *F*.

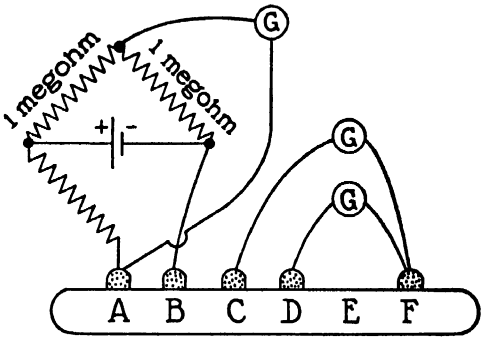

FIG. 4. Showing arrangement when the circuit through *A* and *B* constitutes one arm of an equal-arm Wheatstone bridge. In this case the stimulating E.M.F. does not affect the photographic record directly. Here *G* signifies a string galvanometer with a vacuum tube amplifier. Contacts at *A*, *B*, etc. are made with moist cotton.

DISCUSSION

If distilled water can leach materials out of the cell it would not be surprising if it eventually produced irreversible injury: this appears to be the case in exposures of 3 weeks or more in our experiments. In this connection we may recall the statements by various investigators that water distilled from apparatus consisting entirely of glass or quartz may be toxic.

It is of interest to inquire how the effects of distilled water on irritability are produced. Since the action current depends on the presence of an outwardly directed P.D. across the protoplasm[8] it might be supposed that this P.D. is lowered by the distilled water to a degree which makes the action current impossible.

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[7] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 361.

[8] Osterhout, W. J. V., *Biol. Rev.*, 1931, **6**, 369.

We therefore measured this P.D. in the usual manner[8] by placing Solution *A* at *C* and *D* and then killing *F* with chloroform and measuring the P.D. between *C* and *F* and *D* and *F*. Since this reduces the P.D. of *F* approximately to zero it gives a measure of the P.D. at *C* and *D*. This was on the average about 85 mv.

The measurement was repeated on other cells in which *C* had been treated with distilled water until irritability had disappeared. The P.D. of *C* was then found to be about 120 mv.

This rise in the P.D. across the protoplasm might be explained in various ways. If, for example, salts were leached out of the protoplasm so as to diminish the inwardly directed P.D. across the inner protoplasmic surface the outwardly directed P.D. across this surface (due mostly to potassium in the sap) would appear to increase, unless compensating processes occurred at the outer surface.

This would indicate that the chief effect of the distilled water is on the outer protoplasmic surface and the protoplasm and that the inner surface (adjoining the vacuole) still gives a marked P.D. due chiefly to the potassium in the vacuole.[8]

The action of distilled water on the outer surface of the protoplasm is evidently to leach something out.[9] The nature of the substance will be discussed in a later paper. According to B. Hansteen Cranner[10] and others[11] living cells of plants[12] when placed in contact with distilled water regularly give off certain substances. They regard these as phosphatides but this identification is not confirmed by Steward.[13]

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[9] The result cannot be due to the slight change in osmotic pressure experienced in passing from Solution *A* to distilled water for the same result is obtained when the cells are transferred from pond water or from very dilute Solution *A* to distilled water.

[10] Cranner, B. H., Zur Biochemie und Physiologie der Grenzschichten lebender Pflanzenzellen, Christiania, Grøndahl and Søns, 1922. (Meldinger fra Norges Landbrukshøiskole, 1922, **2**, Nos. 1 and 2.)

[11] Grafe, V., *Biochem. Z.*, 1925, **159**, 445; 1929, **205**, 256; *Beitr. Biol. Pflanz.*, 1928, **16**, 129. Grafe, V., and Horvat, V., *Biochem. Z.*, 1925, **159**, 449. Grafe, V., and Magistris, H., *Biochem. Z.*, 1925, **162**, 366; 1926, **176**, 266; **177**, 16. Grafe, V., and Ose, K., *Biochem. Z.*, 1927, **187**, 102. Grafe, V., and Freund, K., *Beitr. Biol. Pflanz.*, 1928, **16**, 140. Magistris, H., *Biochem. Z.*, 1929, **210**, 85. Magistris, H., and Schäfer, P., *Biochem. Z.*, 1929, **214**, 440. Thierfelder, H., and Klenk, E., Die Chemie der Cerebroside und Phosphatide, Berlin, Julius Springer, 1930.

[12] In some cases at least no injury to the cells appears to be involved.

An alternative explanation might be that the supply of soluble calcium in the cell is decreased by metabolism so rapidly that it falls below the level needed for irritability unless continually renewed from without. This seems improbable in the short time required for these experiments and especially in view of the fact that we find no such deposits of calcium oxalate as occur in many plant cells.

The simplest assumption would appear to be that the cell manufactures one or more substances which may be called collectively *R*. This enters the surfaces and makes possible the normal irritability. In pond water[14] or in Solution *A* this substance dissolves out very slowly so that it is replaced by the cell about as rapidly as it comes out but at certain times of the year the dissolving action becomes more rapid or the production of *R* is slower so that normal irritability disappears. When cells are placed in distilled water the leaching action is so rapid that the outer protoplasmic surface loses *R* more rapidly than it is acquired and in consequence the normal irritability is lost. This effect of distilled water may be largely due to the absence of calcium[15] since we find that the addition of about 0.001 M $CaCl_2$ to distilled water prevents this effect. When irritability has been lost in distilled water it can be restored about as readily in 0.001 M $CaCl_2$ as in Solution *A*. This would be expected if we were dealing with the substances observed by B. Hansteen Cranner since he states that calcium prevents their solution. Loeb[16] suggested that the protoplasmic surface resembles a soap which is made harder by calcium and softer by sodium and potassium. Other observers have noted specific effects of calcium on the protoplasmic surface.[17]

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[13] Steward, F. C., *Biochem. J.*, 1928, **22**, 268; *Brit. J. Exp. Biol.*, 1928–29, **6**, 32.

[14] Cells transferred from pond water to distilled water act like those transferred from Solution *A* to distilled water.

[15] Some of the other bivalent or trivalent cations would no doubt act somewhat like calcium.

[16] Loeb, J., The dynamics of living matter, New York, The Columbia University Press, 1906.

[17] *Cf.* Höber, R., Physikalische Chemie der Zelle und Gewebe, Leipzig, W. Engelman, 6th edition, 1926, 696. Examples will be found in recent work on blastomeres and on microdissection.

In this connection we may recall the injurious effects of lack of calcium in all sorts of organisms, including the phenomena of antagonism. These may depend on the fact that an important function of calcium is to prevent the leaching out of substances from the surface. Apparently only a little calcium is needed for this purpose. A great excess of calcium may prove toxic by acting in some other way. A good illustration of this is found in *Halicystis* as described by Blinks.[18] In sea water the cells show an outwardly directed P.D. of 60 to 80 mv. which quickly disappears when 0.6 M NaCl is substituted for sea water. But when 2.5 parts of 0.4 M $CaCl_2$ are added to 97.5 parts of 0.6 M NaCl the P.D., although falling at first, rises to nearly the normal value. In pure 0.4 M $CaCl_2$ on the other hand it drops approximately to zero and so continues.

It may be remarked in passing that the sap of *Valonia* contains little or no calcium[19] but the sap is probably nearly saturated with *R* so that calcium is not needed to prevent leaching of *R* in the vacuole. Lack of calcium in the external solution soon produces injury.

The fact that anesthesia can be produced by removing something from the cell raises the question whether other cases of anesthesia may be explained in the same way. As a matter of fact one of the earliest theories of anesthesia, that of Bibra and Harless (1847) was precisely this;[20] *i.e.*, that chloroform and ether dissolved out certain substances from the brain.

It is of interest to find that reversible anesthesia can be maintained for a week at a time; this recalls the long periods of anesthesia possible with certain animals.[21]

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[18] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13,** 223.

[19] This seems to be the case with soluble calcium in many flowering plants according to analyses by the senior author. But according to Thoday and Evans (Thoday, D., and Evans, H., *Ann. Bot.*, 1932, **46,** 781) in certain plants soluble calcium and soluble oxalate may exist in different cells. When the sap is extracted mutual precipitation occurs. Hence the analysis will show less than the true amount of soluble calcium. See also Czapek, F., Biochemie der Pflanzen, Jena, Gustav Fischer, 3rd edition, 1925, **3,** 70.

[20] Henderson, V. E., *Physiol. Rev.*, 1930, **10,** 171.

[21] Animals may be anesthetized for several days at a time without permanent injury, *e.g.* tadpoles (Overton, E., Studien über die Narkose, Jena, Gustav Fischer, 1901), frogs (Krogh, A., cited in Winterstein, H., Die Narkose, Berlin, Julius Springer, 2nd edition, 1926, 40; for experiments by Winterstein see *Biochem. Z.*, 1915, **70,** 130), and birds (Ellis, M. M., *J. Pharmacol. and Exp. Therap.*, 1923, **21,** 323).

In this connection we may state that in the late spring it is not uncommon to find cells in the ponds which cannot be stimulated electrically[22] when brought into the laboratory. Apparently this condition may last for weeks in their natural environment. We are not able to change this by keeping them in Solution *A*. It would therefore seem that the difficulty is in the cells themselves which do not produce *R* in normal quantity at this season.

In conclusion we may emphasize that the term anesthesia is here employed, as often in nerve physiology, merely to denote lack of response to electrical stimulation: other effects were not investigated, except that it was noted that protoplasmic streaming continues after leaching with distilled water.

SUMMARY

Cells of *Nitella flexilis* Ag. lose their power to respond to ordinary electrical stimulation after 2 or 3 days in distilled water. It returns after a day or so when they are replaced in their normal environment, in a suitable nutrient solution, or in a dilute solution of $CaCl_2$.

Here anesthesia seems to be produced by removing something from the cell and this raises the question whether other cases of anesthesia may be explained in the same way.

The antagonistic action of calcium, in some cases at least, appears to depend on its power to prevent substances from leaching out of the cell.

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[22] *I.e.* earlier in the season such cells can be stimulated by 50 to 160 mv. but in the late spring they cannot be stimulated by 300 mv. or even more.

ANESTHESIA IN ACID AND ALKALINE SOLUTIONS

By W. J. V. OSTERHOUT and S. E. HILL

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Accepted for publication, July 5, 1933)

We have found[1] that anesthesia is produced in *Nitella* by treatment with distilled water. Our experiments show that the action of distilled water is hastened by adding acid or alkali and retarded by adding calcium.

We can secure the same effects by exposure to 0.0001 M HCl for an hour or to 0.001 M NaOH for 2 or 3 hours as by an exposure of 2 or 3 days to distilled water (all of these effects are fully reversible).

This may be illustrated by describing some typical experiments (all the details are as in the first paper[1] unless otherwise stated[2]).

Effects of Alkali

A group of cells which had been kept for 3 weeks in Solution *A*[1] was transferred to paraffin blocks (Fig. 1) and tested, and all of them responded to electrical stimulation of 160 mv. (*A* responded on break). Solution *A* in Cups *A* and *E* was now replaced by 0.001 M NaOH, and the cells were tested at short intervals. After 75 minutes of extraction with 0.001 M NaOH at *A* and *E*, the cells were tested with 300 mv., with the following results. Of 12 cells

None responded at *A*
12 " " *D* (control spot)
3 " " *E*

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[1] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 87.

[2] Preliminary experiments showed that cells placed in 0.0001 M HCl (pH 4) and 0.001 M NaOH (pH 11) lived 24 hours or longer (the pH value in NaOH fell during the exposure). In applying or removing acid or alkaline solutions there is an effect on P. D. due to the diffusion potential in water of the acid or alkali: the amount of this can be estimated from experiments on dead cells.

The temperature was 23–25°C.

To test whether the effects of NaOH could be reversed by neutralization of the NaOH, Cups *A* and *E* were filled with 0.0001 M HCl and tested by applying 300 mv. a few minutes later. Only one cell responded at *A*. Tested 1 hour later, none responded, and in 5 hours all were dead. Obviously something more than removal of the NaOH is necessary to restore irritability.

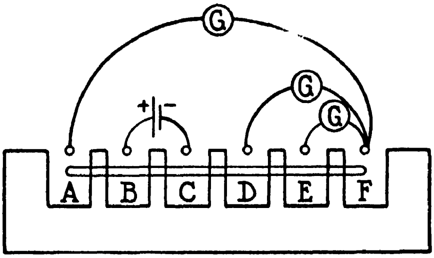

FIG. 1. Diagram of a series of paraffin cups, *A*, *B*, *C*, etc., with a single cell of *Nitella* passing through all of them (in each partition the *Nitella* cell is sealed in with vaseline). Stimulation is applied in the circuit between *B* and *C*. We lead off from *A* to *F*, *D* to *F*, and *E* to *F* through a string galvanometer (*G*) in circuit with a vacuum tube. The whole is covered with a glass plate to prevent evaporation.

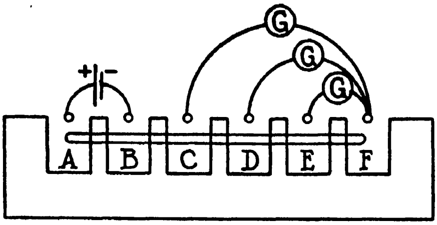

FIG. 2. As in Fig. 1 with a different arrangement of leads.

A second group of 12 cells which had been kept in Solution *A* for 3 weeks was tested and all responded to stimulation by 160 mv. Solution *A* at one point (*C*, Fig. 2) was then replaced with 0.001 M NaOH, which was left in the cups for 3 hours, until no cell responded to stimulation by 300 mv. The 0.001 M NaOH was then replaced by 0.01 M $CaCl_2$, which produced no immediate effect. Left overnight with $CaCl_2$ at *C*, 7 cells out of 12 responded at *C* to stimulation in the circuit between *A* and *B* (200 mv.). The average magnitude of the response was 36 mv., about one-half of the normal value. Partial recovery from the effects of NaOH was thus induced by 0.01 M $CaCl_2$.

A third group of 12 cells, with 0.001 M NaOH at *C*, was stimulated in the circuit between *A* and *B* with 200 mv. at short intervals for 3 hours until irritability disappeared. The 0.001 M NaOH in Cup *C* was then replaced by Solution *A*. Left overnight, 5 cells out of 12 responded at *C* (2 responded to 300 mv., and 3 required 500 mv. for stimulation).

To determine the value of the outwardly directed potential in cells which had lost their irritability in 0.001 M NaOH, a group of 15 cells was treated by immersion in a bath of the alkali for 2 hours. The potential was then measured, using saturated calomel electrodes, with 0.001 M NaOH on one end of the cell, and 0.001 M NaOH saturated with chloroform at the other. The outwardly directed potential at the living end had an average value of 112 mv. 15 control cells in Solution *A* exhibited an average potential of 76 mv. (when one end was killed with Solution *A* saturated with chloroform). It would seem that the P.D. across the protoplasm had been somewhat increased by the treatment, as is the case with distilled water.[1]

Effects of Acid

We had observed in earlier unreported experiments that *Chara* kept for 48 hours in pond water nearly saturated with CO_2 lost its irritability, and had an outwardly directed potential about 15 per cent above the value for cells kept in normal pond water. The control cells in normal pond water responded to electrical stimulation. Another group of cells kept for 48 hours in a mixture of 0.01 M $NaHCO_3$ and CO_2 at pH 6.0 lost their irritability, and retained the normal P.D. across the protoplasm.

A group of 12 *Nitella* cells which had been kept in Solution *A* for 3 weeks was tested with Solution *A* at all contacts, and responded to stimulation of 160 mv. Solution *A* in Cups *A* and *E*, (Fig. 1) was now replaced with 0.0001 M HCl, and the cells were tested after 1 hour with 300 mv. Every cell responded at *D*, which had been in contact with Solution *A* throughout. Four responded slightly at *A*, and none at *E*. The 0.0001 M HCl at *A* was now replaced by 0.001 M $CaCl_2$ and the 0.0001 M HCl at *E* was replaced by Solution *A*. 1½ hours later the cells were tested by stimulation with 200 mv. at *B*. Every cell responded at *D*, 10 responded at *A*, and 10 responded at *E*. Thus Solution *A* and 0.001 M $CaCl_2$ seem to be equally efficacious in restoring irritability which has been removed by leaching with 0.0001 M HCl.

In another experiment Solution *A* in Cup *D* was replaced by 0.0001 M HCl. The other cups contained Solution *A*, in which the cells had been kept for 3 weeks. The cell was stimulated (160 mv.) just before putting 0.0001 M HCl at *D*, then immediately after the change, and thereafter at intervals of about 2 minutes for 30 minutes until irritability disappeared (*i.e.*, no response to 400 mv.). The magnitude of the response gradually fell off, the reduction being most marked in the second peak. The second peak disappeared in about 5 minutes, and response failed altogether after 30 minutes.

To determine the value of the outwardly directed potential of a spot which has lost its irritability in 0.0001 M HCl, Solution *A* in Cups *D* and *E* (Fig. 2) was replaced by 0.0001 M HCl. 2 hours later *F* was killed with chloroform. The average value of 10 cells in contact with Solution *A* at *C* was then 66 mv., and the average value of 20 spots in contact with 0.0001 M HCl was 82.5 mv. There is thus a rise in the P.D. across the protoplasm when the cells are leached with 0.0001 M HCl, similar to that when they are leached with distilled water[1] and with alkali.

DISCUSSION

It thus appears that the action of distilled water is hastened by adding acid or alkali. It has been shown previously[1] that it is retarded by calcium. These may all be effects of leaching substances out of the cell. It is of interest to note that Magistris and Schäfer[3] found that the action of distilled water in leaching substances out of plant cells was similarly hastened by the addition of acid and alkali and retarded by calcium.

Alternative explanations are direct action of acid or alkali and the absence of calcium (which has been previously[1] discussed) but if lack of calcium alone were the cause acid should not increase the effect.

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[3] *Cf.* Magistris, H., and Schäfer, P., *Biochem. Z.*, Berlin, 1929, **214**, 440. They regard these substances as phosphatides but this identification is not confirmed by Steward (Steward, F. C., *Biochem. J.*, London, 1928, **22**, 268; *Brit. J. Exp. Biol.*, 1928–29, **6**, 32).

It is possible that some of the reported cases of narcosis in various organisms induced by acid or alkali are due to leaching substances out of the cell.

SUMMARY

The action of distilled water in producing anesthesia (loss of response to electrical stimulation) in *Nitella* is hastened by the addition of acid and alkali and retarded by the addition of calcium. The loss of irritability is fully reversible.

REVERSIBLE LOSS OF THE POTASSIUM EFFECT IN DISTILLED WATER

By W. J. V. OSTERHOUT AND S. E. HILL

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Accepted for publication, June 30, 1933)

Not only does distilled water take away the irritability[1] of *Nitella*[2] but it also changes its behavior toward potassium. In normal cells potassium is strongly negative to sodium: this will be called for convenience the potassium effect. After sufficient exposure to distilled water this effect disappears but it can be restored by returning the cells to their normal environment or to nutrient solutions. This change in the protoplasm seems to be chiefly confined to its outer surface.

These facts may be illustrated by citing a few typical experiments.[3]

A group of cells was divided, alternate cells being placed in distilled water and in a nutrient solution called Solution *A*.[1] 3 days later the cells were taken out of Solution *A* and placed on paraffin blocks,[4] being surrounded by moist air except at the contacts *C*, *D*, *E*, etc. (Fig. 1). At first Solution *A* was placed at all contacts. Then Solution *A* was replaced at *C* by 0.01 M NaCl which made little change in potential. Substitution of 0.01 M KCl for 0.01 M NaCl caused the potential at *C* to become 86 mv. more negative, a normal potassium effect. In the other group of cells, which had been kept in distilled water 3 days, distilled water at *C* was replaced by 0.01 M NaCl and then by 0.01 M KCl. The change in potential was much less, K becoming only 20 mv. negative to Na.

In a similar experiment with a different lot of cells 0.01 M KCl was 64 mv. negative to 0.01 M NaCl on the control cells in Solution *A*, but the cells leached in distilled water for 4 days made no discrimination between K and Na.

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[1] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 87.

[2] This is *Nitella flexilis* Ag., the species used in previous experiments in this laboratory.

[3] The experiments were performed at 21–23°C.

[4] For technique see footnote 1.

It would appear therefore that distilled water can leach out of the cell something which is responsible for the potassium effect.

It is of interest to know the potential across the protoplasm in cells which do not show the potassium effect. A group of cells was leached 6 days in distilled water. Then 0.01 M NaCl was placed on contacts *C* and *F*, Fig. 1. The solution at *F* was then changed to 0.01 M KCl, which made no great difference in potential, *F* becoming 12 mv. more negative. The 0.01 M KCl was then replaced by 0.01 M KCl saturated with chloroform, which reduced the potential at *F* approximately to zero: *C*, in contact with 0.01 M NaCl, was then 110 mv. positive to *E*. Since *C* and *F* were previously at nearly the same potential it is evident that *F* when in contact with 0.01 M KCl (without chloroform) had an outwardly directed P.D. of 98 mv. which is about what would be expected in a cell taken from Solution *A* and placed in contact with 0.01 M NaCl. In other words the P.D. across the protoplasm had not been lessened by the treatment with distilled water.

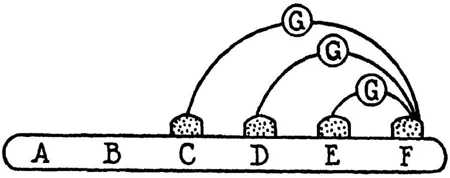

FIG. 1. Arrangement of *Nitella* cells for rapid testing of potassium effects. Contact with the cell is made by means of wads of cotton dipped in the solutions. *G* denotes a string galvanometer in series with a vacuum tube.

In a previous paper[1] it was reported that local areas of the cell could be anesthetized by distilled water. It is of interest to determine whether the potassium effect shows a similar behavior.

Cells of *Nitella* which had been kept for several days in Solution *A* were placed in paraffin cups[4] separated by paraffin partitions (Fig. 2). Solution *A* was applied at *A*, *B*, *C*, *D*, *E*, and *F*. We found only a small P.D. between *F* and the other cups. We then substituted 0.01 M NaCl for tap water at *C* and *D*, which made little change at either spot. We then applied 0.01 M KCl at *D*. This produced a great change: before applying KCl *D* was 4.0 mv. positive to *F* but afterward it was 64 mv. negative to *F*, a normal potassium effect.

We then applied distilled water at *C* and *D* for 2 days. On substituting 0.01 M NaCl for distilled water at *C* and at *D* very little change was observed. We then substituted 0.01 M KCl for 0.01 M NaCl at *C* and *D*. This substitution which formerly produced a great change now had very little effect, 0.01 M KCl becoming only 14 mv. negative to 0.01 M NaCl. Hence it would appear that something had been leached out of *C* and *D* so that they no longer behaved normally toward KCl. In other words the normal potassium effect had disappeared as the result of the exposure to distilled water.

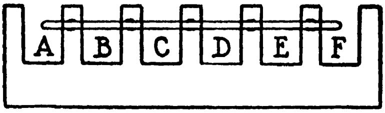

FIG. 2. Diagram of a series of paraffin cups *A*, *B*, *C*, etc., with a single cell of *Nitella* passing through all of them (in each partition the *Nitella* cell is sealed in with vaseline). We lead off from one cup to another through a string galvanometer in series with a vacuum tube.

In order to ascertain to what extent this effect of distilled water is reversible the experiment was repeated. After the leaching of *C* and *D* by distilled water 0.01 M NaCl was applied. As before this produced little change nor did the substitution of KCl for NaCl. The KCl was then replaced by Solution *A*. The P.D. between *C* and *F* was then 11 mv., *C* being positive: that between *D* and *F* was 7 mv.

After 24 hours Solution *A* was replaced by 0.01 M NaCl which made little change. Then 0.01 M KCl was substituted for NaCl. This made a great change, *C* becoming 64 mv. and *D* 55 mv. more negative. It would therefore seem that the normal state of the protoplasm had been restored, as the potassium effect before leaching was 68 mv. By applying Solution *A* saturated with chloroform at *F* we found that the P.D. across the protoplasm at *C* was 3 mv. and at *D* 4 mv. which is about the usual value for a normal spot in contact with 0.01 M KCl.

It therefore appears that Solution *A* can restore the P.D. across the protoplasm to normal after it has been leached by distilled water.

All this could be easily explained by saying that the cell constantly produces certain substances, which may collectively be called *R*, which move into the protoplasmic surfaces and ensure its normal behavior. When *R* is leached out by distilled water faster than it is produced the behavior becomes abnormal. But this does not happen in the presence of tap or pond water or of Solution *A*, presumably because in these cases *R* is produced faster than it is leached away. It is quite possible that calcium is an important factor in this situation and that it tends to prevent the rapid leaching of *R*. This has been discussed in a previous paper.[1]

The leaching effect does not appear to affect the inner protoplasmic surface greatly nor to lower the concentration of potassium in the sap because the P.D. across the protoplasm does not fall off. This P.D. appears to be due for the most part to the action of the potassium salts in the vacuole on the inner protoplasmic surface, as explained in previous papers.[5]

It may be added that the loss of the potassium effect appears to precede the loss of irritability. This will be discussed in later papers. It was observed that the treatment did not stop the protoplasmic motion.

It is interesting to note that cells collected in June (a season of active growth[6]) cannot as a rule be stimulated electrically[1] and that the potassium effect is greatly reduced or altogether lacking. Placing them in Solution *A* does not alter this situation. Hence we conclude that it does not arise from a change in the pond water but rather from a change in the cells which probably are not producing the normal amount of *R* at this season.

SUMMARY

Not only does distilled water take away the irritability of *Nitella* but it also changes its behavior toward potassium. In normal cells potassium is strongly negative to sodium but after sufficient exposure to distilled water this effect disappears. It can be restored by returning the cells to their normal environment or to a suitable nutrient solution. This change in the protoplasm seems to be chiefly in its outer surface.

[5] *Cf.* Osterhout, W. J. V., *Biol. Rev.*, 1931, 6, 369.

[6] The cells used in the experiments may be mature or nearly so and hence need not be actively growing.

PROTOPLASMIC POTENTIALS IN HALICYSTIS

III. The Effects of Ammonia

By L. R. BLINKS

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Accepted for publication, July 1, 1933)

The large potential difference measurable across the protoplasm of impaled multinucleate cells of the alga *Halicystis* has been described in preceding papers.[1,2] This is called "protoplasmic" since it is, to a large extent, independent of concentration gradients between sea water and the vacuolar sap. Thus a large P.D. persists when natural or artificial sap is applied externally, both in the species containing much KCl and in that containing little. Dilution and concentration of the sea water, or of many of its constituents, provided a reasonable physiological balance of salts is maintained, also have little effect, after certain transient changes have occurred. However, such applications at the outer surface may not immediately affect potentials at the inner surface, or within the protoplasm itself. To study these, internal changes must be produced. The effect of naturally differing cell saps on the vacuolar surface has already been noted.[2] There remain as experimental changes: (1) direct alteration of the cell sap by injection or perfusion; (2) the penetration of substances into the cell from the sea water.

Both of these methods have been employed, with marked effects upon the observed P.D. The results with ammonia, representing the penetration of a substance into the cell, are reported in the present paper. Being technically simpler to produce than the changes by sap perfusion, they were completed first. The results, however, are inherently more complex, since they involve not only the changes known to occur in the sap, but also unknown changes in the protoplasm across which the ammonia must pass to reach the vacuole. Some of the results of direct perfusion which bear upon the interpre-

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[1] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 223.

[2] Blinks, L. R., *J. Gen. Physiol.*, 1932–33, **16**, 147.

tation are therefore indicated, as in the paper on the effects of KCl.[2] More complete description of the technique of perfusion and the principal results will appear in a forthcoming paper.

Methods

The cells were held rigidly between a glass ring below, and a glass tube, constricted to a capillary, inserted into the cell from above, as in Fig. 1. The tube and capillary, filled with artificial sap, formed the circuit from the vacuole to a KCl-agar bridge, communicating with a saturated KCl-calomel electrode. External solutions were changed without disturbing the cell by replacing vessels from below, or directing a stream of solution on the tube a short distance above the cell. This flowed down the tube and over the cell, dropping off below, making contact with a second KCl-agar bridge opening just beside or below the cell. A second saturated KCl-calomel electrode completed the electrical circuit to the measuring instruments, a potentiometer and galvanometer. The latter could be employed without prejudice to the results, since it was found that *Halicystis* cells can withstand quite appreciable current drain without decrease of potential. Except during rapid changes of P.D., however, the circuit was kept compensated and no current passed through the cell. No different results were found when a vacuum tube electrometer, or a Compton electrometer, was used as a nullpoint indicator. Points of balance could easily be obtained at 15 second intervals; usually 1 minute intervals were adequate to follow the changes here described.

The potential plotted is that of the outer surface or solution (usually sea water). When this is positive, the positive current tends to flow outward across the protoplasm to the measuring instrument; when negative, it tends to flow inward. The positive sign is below the zero line, in conformity with the convention for *Valonia* and *Nitella* in this laboratory. This is the reverse of the plotting in the first paper[1] of this series, but is followed in subsequent articles.

pH values of sap and sea water were determined colorimetrically with allowance for salt errors, the buffers used for comparison being checked with the quinhydrone electrode below pH 8.0. Ammonia estimations in the sap were made by Nessler test. The ammonia content of the sea water was regulated by adding measured quantities of NH_4Cl from a stock solution for each experiment, observing, and if necessary adjusting, the pH (by adding NaOH or HCl).

Sap was withdrawn from the cells by inserting a glass tube drawn into a fine point, rinsing and wiping the cells well to avoid contamination from sea water. The pH, determined directly in these tubes, with a minimum of exposure to air, was found to be very much lower than originally reported by Blinks and Jacques;[3]

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[3] *Halicystis Osterhoutii*, described by Blinks, L. R., and Blinks, A. H., *Bull. Torrey Bot. Club*, 1930–31, **57**, 389; sap analysis by Blinks, L. R., and Jacques, A. G., *J. Gen. Physiol.*, 1929–30, **13**, 733.

namely, 5.0 instead of 6.1. The earlier determination was on a large mixed sample extracted from many cells and exposed to the air during collection. Hollenberg[4] found the sap of *H. ovalis* to have a pH of 5.4; *Halicystis* sap is thus considerably more acid than that of *Valonia*.

The temperatures varied from winter values of 18°C. to summer ones of about 25°C. This variation had very little effect upon the P.D. across the protoplasm at the different times, the summer values averaging possibly a millivolt or two lower than the winter ones. There may be other reasons for this, such as the onset of the reproductive period in the summer. The variation of temperature in a single experiment was seldom more than 1°C.

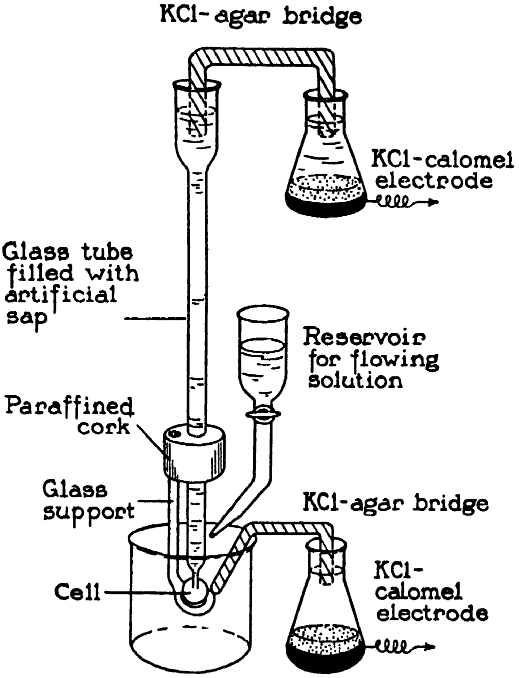

FIG. 1. Diagram of arrangement for holding impaled cells of *Halicystis*, with connection to calomel electrodes.

Variation of illumination has also small effect upon the normal potentials, which hold up for several days in complete darkness. In the presence of NH_4Cl in the sea water, however, illumination has a perceptible or even very marked effect under proper conditions. In ordinary diffuse north light of the laboratory this was negligible, but on bright days, passing clouds often changed the P.D. appreciably. This is evidently due to the photosynthetic extraction of CO_2 from the sea water around the cell, raising the pH and so influencing the dissociation of ammonia. It is hoped to study this effect more fully, as a sensitive electrical indicator of photosynthesis.

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[4] *Halicystis ovalis*, morphology treated by Smith, G. M., in Contributions to marine biology, Stanford University Press, 1930, 222. Sap analysis by Brooks, S. C., *Proc. Soc. Exp. Biol. and Med.*, 1929–30, **27**, 209; Hollenberg, G. J., *J. Gen. Physiol.*, 1931–32, **15**, 651.

The results with *Halicystis Osterhoutii*,[3] of Bermuda, are chiefly described in the present paper, since it is with this species that the experiments with sap perfusion have been performed. It is also perhaps of greater intrinsic interest, since its cell sap differs so slightly from the sea water; the potential is therefore almost entirely protoplasmic, and not enhanced by a KCl gradient.[2] In a few cases, the almost identical results obtained with *H. ovalis*,[4] of California, will be referred to. The chief difference is the usually higher threshold of NH_4Cl necessary for potential reversal in the latter species.

The Reversal of P.D. by Ammonia

The normal concentration of total ammonia ($NH^+_4 + NH_4OH + NH_3$) in Bermuda sea water is below 0.00001 M. Up to about 0.0001 M NH_4Cl may be added, at the normal pH, 8.1, of the sea water, without appreciable effect upon the P.D. across the protoplasm of *Halicystis Osterhoutii* which averages remarkably close to 68 mv., outside positive.[2] But at a threshold varying between 0.0005 M and 0.002 M, often at about 0.001 M NH_4Cl, a striking change occurs. The P.D. rapidly reverses, to about 30 or 40 mv. negative, and remains reversed (with fluctuations) as long as the exposure to the ammonia continues. Higher concentrations increase the negative P.D. somewhat; a return to ordinary sea water causes recovery of positivity. The entire process is completely reversible, and may be repeated almost without limit if the ammonia exposures do not last too long; *e.g.*, more than an hour or two at a time.

A characteristic time curve of the reversal and recovery process at about the threshold concentration of NH_4Cl is shown in Fig. 2. When the ammonia is first applied there is a small notch or cusp (*a*), during which the P.D. decreases a few millivolts, then recovers nearly to its original value (*b*). Here it remains a few minutes, then begins to decrease slowly to about 40 mv. positive. After this the decrease becomes much faster and the P.D. rapidly reverses (*c*) to about 40 mv. negative. At this point there almost invariably occurs a reverse cusp (*d*), then a return to an irregularly wavering negative value (*e*). When ordinary sea water is again replaced, a positive P.D. is quickly recovered (*f*), usually with a period of enhanced positivity (*g*) up to 75 mv. or more, before the normal value (*h*) is regained.

Fig. 2 is entirely typical of dozens of observed reversals at about the threshold concentration of NH_4Cl. The actual speed of reversal (and the negative P.D. attained) varies somewhat from cell to cell, as does the threshold itself. When higher concentrations of NH_4Cl are applied, the reversal becomes much quicker, as shown in Fig. 3. The curve becomes very abrupt and almost rectangular at high concentrations; the reversed P.D. may also become temporarily as high as 90 to 100 mv. negative; *i.e.*, about as high, although with reversed sign, as the greatest positive values so far produced (with alkaline sea water). Usually, however, the negative P.D. does not greatly exceed

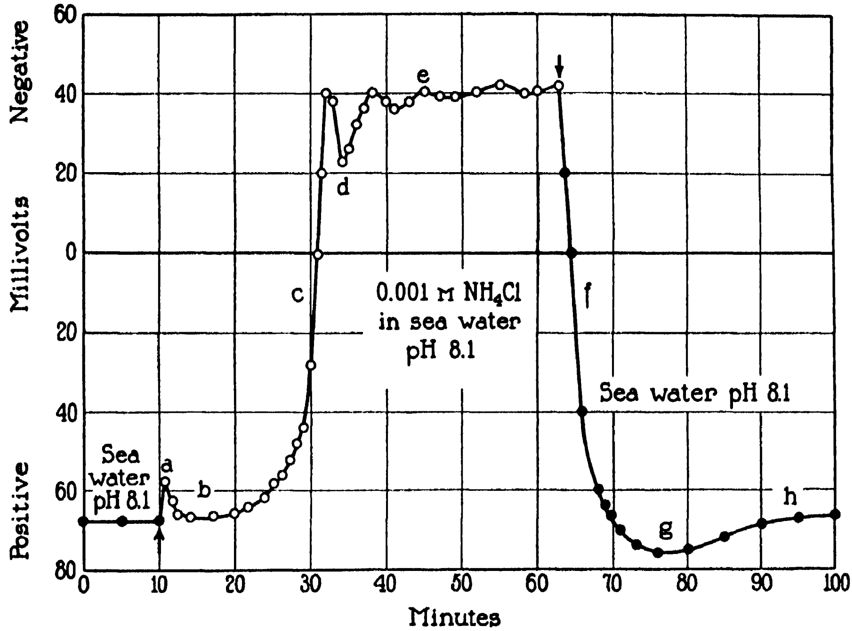

FIG. 2. Time course of P.D. change in cell of *Halicystis Osterhoutii* exposed to sea water containing 0.001 M NH_4Cl at pH 8.1. Arrows indicate change of solution.

70 mv., again about the average for the positive values in normal sea water.

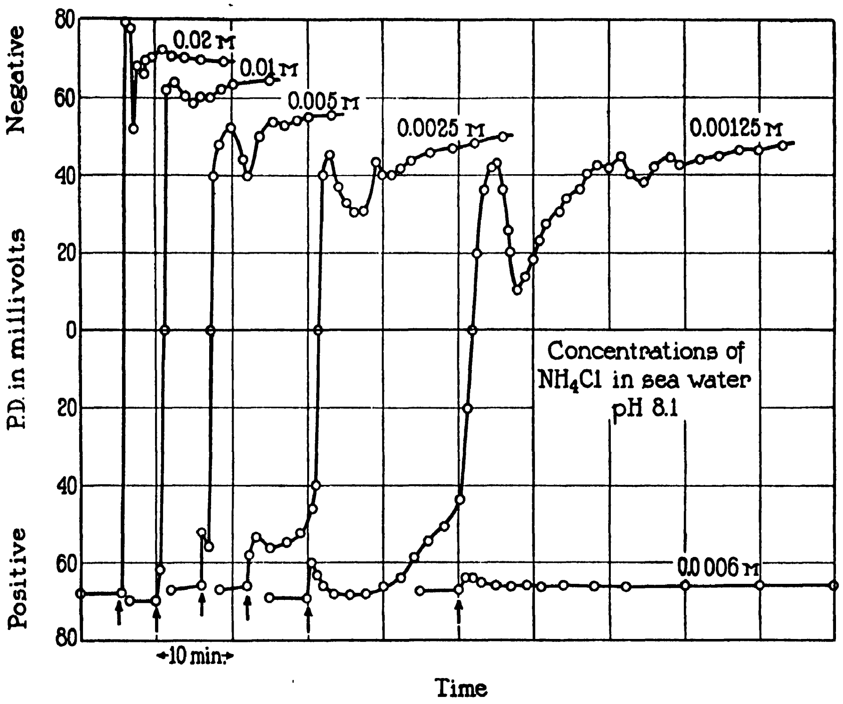

FIG. 3. Time course of P.D. change in cells of *Halicystis Osterhoutii* exposed to the indicated concentrations of NH_4Cl in sea water at pH 8.1. The rise becomes much more abrupt with increasing concentrations. The recovery curves in normal sea water are omitted to prevent overlapping but closely resemble the recovery in Fig. 2. Arrows indicate the appication of sea water containing ammonia.

The Relation of NH_4Cl Concentration to P.D.

It is evident that although the P.D. has a very definite relation to the NH_4Cl concentration in the sea water, it is distinctly not proportional, but rather in the nature of an "all or none" response: below a critical concentration, there is very little change of P.D.; at the threshold a reversal amounting to a change of 100 mv. or more; and then again above the threshold only relatively small increases of P.D. This is shown for a single cell with a series of increasing concentrations in Fig. 4. Furthermore when the concentrations are decreased, it is seen that the threshold for recovery is at a lower NH_4Cl concentration than for original reversal. Thus the reversed potential is maintained with only half the NH_4Cl concentration necessary originally to cause reversal; only at about one-fourth of the original threshold value is the positive P.D. recovered, and then with a slower curve than in ordinary sea water.

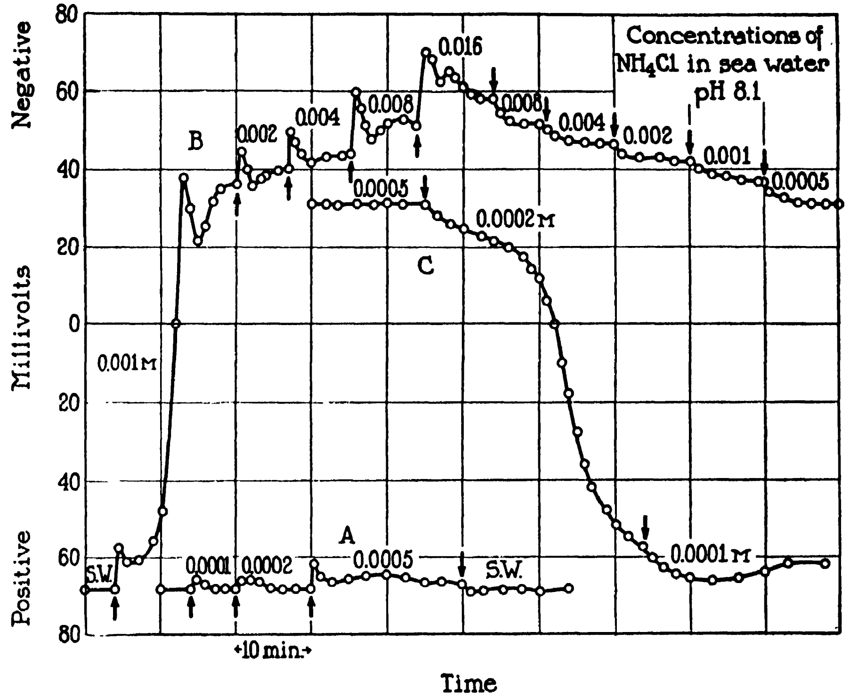

FIG. 4. Time course of P.D. change in cells of *Halicystis Osterhoutii* exposed to sea water at pH 8.1 containing first increasing and then decreasing concentrations of NH_4Cl. Curve *A* is for concentrations below the threshold (in this case below 0.001 M), Curve *B* shows the reversal of P.D. at 0.001 M and the effect of successively doubled NH_4Cl concentrations, followed by halving to 0.0005 M. Curve *C* shows recovery of positive P.D. at 0.0002 M NH_4Cl. Arrows indicate change of solution.

This is something in the nature of hysteresis, giving two curves, one for increasing, the other for decreasing NH_4Cl concentrations. For the reversed P.D. there are also two points, at least, for each NH_4Cl concentration: the maximum reached first on reversal, before the cusp (*d* of Fig. 2) and an average of the wavering plateau (*e*). No single curve of P.D. against NH_4Cl concentration can thus be plotted. There are three which could be used: (*A*) the highest values reached during increase of concentration; (*B*) the lowest values reached during increased concentrations; and (*C*) the steady values reached after decreased concentrations. The latter are probably the most reliable

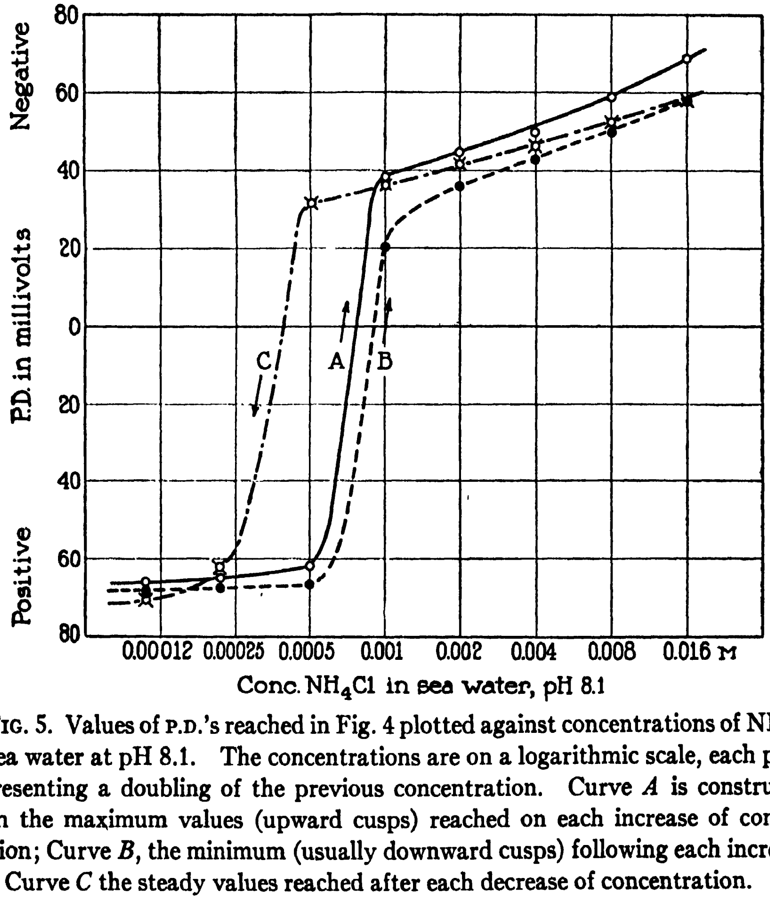

FIG. 5. Values of P.D.'s reached in Fig. 4 plotted against concentrations of NH_4Cl in sea water at pH 8.1. The concentrations are on a logarithmic scale, each point representing a doubling of the previous concentration. Curve *A* is constructed from the maximum values (upward cusps) reached on each increase of concentration; Curve *B*, the minimum (usually downward cusps) following each increase; and Curve *C* the steady values reached after each decrease of concentration. The last are probably the most reliable values. The lag or hysteresis of threshold between increase and decrease of concentrations is to be noted. Upward arrows indicate the series with increasing concentrations of NH_4Cl; the downward arrow that with decreasing concentrations.

values. All of these are plotted, for the cell of Fig. 4, in Fig. 5. Such a plot for a single cell is more significant than an average or composite plot for many cells, as showing more sharply the reversal at a critical concentration. It is, moreover, quite characteristic for any other cell, with a slight shift of the critical concentrations and of actual P.D. values.

The Relative Rôles of Ammonium Ions and Undissociated Ammonia

It might be concluded from Fig. 5 alone that we are here dealing with no ordinary ionic concentration effects, so different is this relation from the usual one relating P.D. to salt concentration, *i.e.* in straight logarithmic ratio (*cf.* Damon's results with Na and K in *Valonia*[5]). There is, however, still more direct evidence as to the slight effect of ammonium ions on the P.D. of *Halicystis*. The basic dissociation constant[6] of ammonia being approximately $10^{-4.7}$, ammonium salt will be 50 per cent dissociated at pH 9.3. At the ordinary pH of sea water, 8.1, it will therefore be over 95 per cent dissociated,

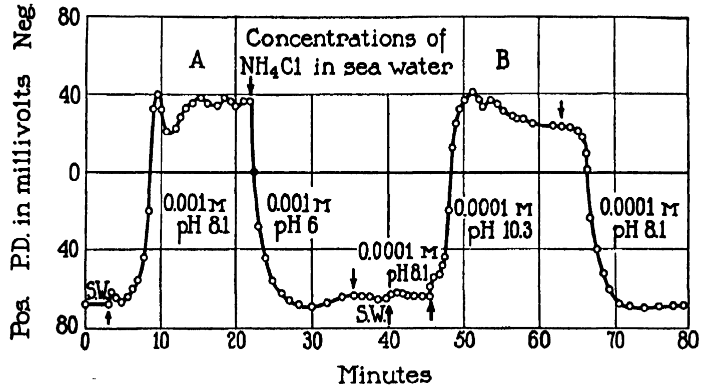

FIG. 6. Change of P.D. across protoplasm of *Halicystis Osterhoutii* showing effect of change of pH. In *A* reversal of P.D. occurring with 0.001 M NH_4Cl at pH 8.1, is inhibited by lowering the pH to 6, and positive P.D. regained. In *B* 0.0001 M NH_4Cl is insufficient to cause reversal at pH 8.1, but is effective when the pH is raised to 10.3. Recovery of positive P.D. then occurs when pH is again lowered to 8.1. Arrows indicate change of solutions.

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[5] Damon, E. B., *J. Gen. Physiol.*, 1929–30, **13**, 445; 1932–33, **16**, 375.

[6] This is the value commonly assumed for the dissociation constant of ammonia in dilute solution. The greater ionic strength of sea water will tend to increase the ionization of ammonia, but the change was found (Cooper, W. C., Jr., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 117) to be slight, shifting the pK_{ob} from 9.3 to 9.5. This, as well as the difference between concentrations and activities of the substances concerned, may be neglected for the purposes in hand.

and at pH 5, nearly 100 per cent dissociated. For a given NH_4Cl concentration the concentration of ammonium ions will be increased about 5 per cent when sea water is acidified from pH 8.1 to pH 5; but the concentration of *undissociated* ammonia (NH_3 or NH_4OH) will be 1000 times as high at pH 8 as at pH 5, and at pH 10.3 will be over 100 times as great as at pH 8.1. Changing the pH over this range with definite NH_4Cl concentrations should therefore give information on the relative rôle of ammonium ion and undissociated ammonia. Two such experiments are shown in Fig. 6. When reversal is obtained with 0.001 M NH_4Cl at pH 8.1, lowering the pH to 6 causes recovery of positive values; conversely when 0.0001 M NH_4Cl is applied at pH 8.1 it is insufficient to cause reversal, but if the pH is raised to 10.3 good reversal occurs. The relative inactivity of ammonium *ions* in altering the P.D. of *Halicystis* is perhaps most strikingly seen in Fig. 7. Here the sea water (without NH_4Cl) was first acidified to pH 5, giving a typical cusp, presumably due to the diffusion of hydrogen ions into the protoplasm. Then 0.1 M NH_4Cl in sea water of pH 5 was substituted; the additional effect is very slight and reversal does not occur. Indeed, concentrations of NH_4Cl as high as 0.5 M, *i.e.* NH_4Cl entirely substituted for NaCl in van't Hoff artificial sea water, at pH 5 have been applied to some cells of rather

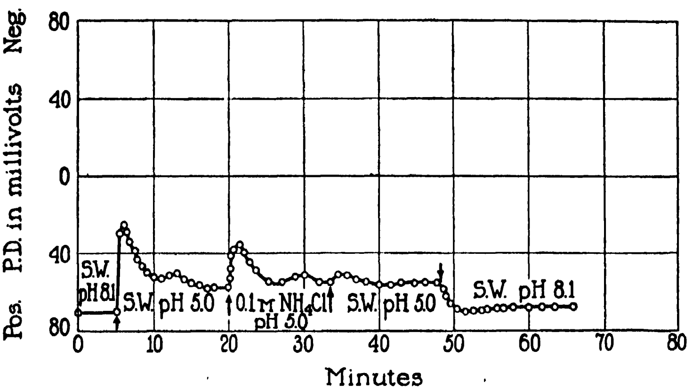

FIG. 7. Variations of P.D. across the protoplasm of *Halicystis Osterhoutii.* Sea water of pH 5 is first applied, producing a characteristic cusp. Sea water containing 0.1 M NH_4Cl at pH 5 is then applied. There is no reversal of P.D. pH 5 sea water and pH 8 sea water then follows, with positive P.D. throughout. Arrows indicate change of solution.

high threshold without reversing the P.D. On the other hand the threshold at pH 10.3 lies between 0.00005 and 0.0001 M NH_4Cl. Since at this pH 95 per cent of the ammonium salt is present as undissociated ammonia these concentrations may be taken as essentially the actual threshold values for NH_3(or NH_4OH). They are in good agreement with the values at pH 8.1 where only about 1/20 as much undissociated ammonia exists, and the threshold of total NH_4Cl concentration lies between 0.001 and 0.002 M; *i.e.*, about 20 times as high. We may conclude that the reversal of potential depends upon the concentration of undissociated ammonia rather than that of ammonium ions in the sea water applied to the cells. But it is again not *proportional* to such ammonia; for the threshold "all or none" effect still holds. We must therefore ask what internal changes may be produced by the entrance of undissociated ammonia, which might account for the abrupt reversal of P.D. at a particular NH_3 concentration.

Internal Effects

The ease with which ammonia enters living cells is one of the well established facts of permeability studies.[7] Not only its tendency to enter more at high pH values, but its observed effects (increase of internal pH in the cell) indicate that it enters as base (NH_4OH) rather than as salt (NH_4Cl), and probably as undissociated ammonia (NH_3) although it could of course enter as the ion pair (NH_4) and (OH). *Halicystis* shows no exception to this tendency; with the increase of undissociated ammonia in the sea water applied to cells, both the pH and the ammonia content of *Halicystis* sap increase.

(*a*) *Increase of Ammonium Salts.*—It seems unlikely that the increase of ammonium salts as such in the cell produces the observed effect on P.D. If the P.D. were due to the mobility of ammonium ions it is difficult to imagine any mechanism or system which would give rise at the threshold to any very sudden increase of ammonium inside (necessarily nearly 50-fold to give 100 mv. potential change). Nor is such a sudden rise detected in the sap. Instead, the total ammonium increases in a regular manner, apparently much as in

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[7] *Cf.* Cooper and Osterhout,[6] and previous experiments of others cited in footnote 2 of their paper.

Valonia.[6] Significant figures for the increase in *Halicystis* cannot be given, because in the cells available for such experiments a considerable and variable amount of ammonium salt (up to 0.005 or even 0.01 M by Nessler test) was already present in the sap,[8] rendering any accumulation study uncertain. But this natural presence of ammonium salts in the sap is in itself an indication that ammonium ions are not concerned in the reversal. If it be assumed that the P.D. suddenly reversed above a critical concentration of ammonium salt in the sap, this should occur sooner, and at a lower threshold concentration of NH_4Cl outside, in those cells already containing considerable ammonium. This is not the case, the threshold being no lower for such cells than for those containing little or no ammonium. If anything, it is a little higher.

Finally, the ammonium salt content of the sap may be experimentally increased, by the method of vacuolar perfusion which will be described in a later paper. Concentrations of NH_4Cl as high as 0.1 M or even 0.5 M have been thus produced in the sap, without causing a reversal of potential, as long as the normal pH of the sap was maintained. In fact the positive P.D. was slightly increased, as with perfusion of KCl.[2] P.D. reversal does not therefore seem to be due to the increase of ammonium ions in the sap.

(*b*) *Increase of pH.*—At first glance, the S shape of the NH_4Cl-P.D. curve (Fig. 5) suggests that it might be explained as an electrometric titration curve, on the assumption that the inner surface of the protoplasm acts like a hydrogen electrode or is a membrane like a glass electrode, responding directly by changes of P.D. to changes of pH produced by the entrance of ammonia. Each increase of NH_3 outside might be like an increment of base in a titration, entering and neutralizing a certain portion of the cell's acids. The great change of P.D. at the reversal point would correspond to the neutralization point, the flatter ends to the smaller changes of pH at either side of neutralization.

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[8] In this respect these cells differed remarkably from those used for the sap analysis previously reported (Blinks and Jacques[3]) which showed practically no ammonia. The reason for this is apparently the age and condition of the cells. Those used for the previous sap analyses were all very young, small cells collected early in the spring before reproduction had occurred and probably of the same year's growth. Those available for the present work were collected later in the spring, and in the fall, and had all undergone several or many reproductive periods. In this species the gametes are frequently not discharged normally to the exterior, but escape into the vacuole, where they swim for a while in the sap, then sink to the bottom in a dark mass.[3] This mass apparently breaks down, with ammonia as one of its products, since cells containing such masses almost invariably have ammonia in the sap. The presence of this ammonia seems in no way toxic; cells from cultures containing it have lived normally in the laboratory for well over a year, growing greatly in size, and reproducing again in the spring at exactly the same day as freshly collected cells (although entirely isolated and without change of sea water for months).

Similarly, on this assumption, Fig. 2 could be interpreted as the time course of such a titration performed with a regularly increasing amount of base—in this case the NH_3 diffusing constantly into the cell as a result of its concentration gradient.

However, neither theory nor facts bear out this suggestion. In the first place we are not performing a titration when we increase the concentration of NH_4Cl in the sea water. We are correspondingly increasing the concentration of undissociated base but the total *amount* depends on the volume of sea water. In these experiments this volume is so much greater than that of the cells that it may be considered infinite; the sea water is also renewed from time to time, or a constant flow is maintained. Therefore at any given concentration NH_3 will continue to enter the cell until its activity is as great inside as outside. In both sap and sea water the equilibrium formula for this would be

$$(NH_3) = \frac{(NH_4^+)(OH)}{K_b} \tag{1}$$

or

$$\log (NH_3) = \log (NH_4^+) + pH - pK_{ab} \tag{2}$$

where (NH_4^+) is the activity of ammonium salt or ions; and pK_{ab} $(= pK_w - pK_b)$ for ammonia[6] lying at pH 9.3. For any constant (NH_4) concentration, therefore:

$$pH \propto \log (NH_3). \tag{3}$$

In other words, when log (NH_3) increases in the sap, the pH will rise proportionally.

The experiments are in good agreement with this expectation.

Fig. 8 shows the actual course of pH change in the sap of *Halicystis* cells exposed to varying concentrations of NH_4Cl over the range well above and below the reversal threshold. For each concentration of NH_4Cl the pH of the sap rises to a nearly constant value during the course of 60 to 120 minutes.[9] When the constant or apparent equilibrium pH value is plotted against the log of outside NH_4Cl concentration (or NH_3, taken as 5 per cent of NH_4Cl at pH 8.1) the essentially straight line of Fig. 9 results.

The plot does not have the full proportionality of 1 to 1 demanded by Equation 2, but is more nearly 4 to 5, the pH of the sap not increasing as fast as the NH_3 outside. Several factors might contribute to this. Probably the sampling of the whole sap, even after 2 hours' penetration of NH_3, does not truly represent the pH just within the protoplasm, which really governs the equilibrium; the same applies to the supposed pH of the sea water just outside the cell. The NH_3

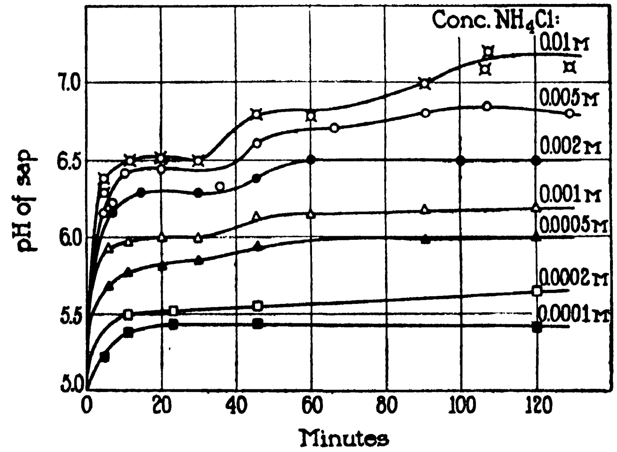

Fig. 8. pH of extracted sap of *Halicystis Osterhoutii* after exposure of cells to indicated concentrations of NH_4Cl (from 0.0001 M to 0.01 M) in sea water of pH 8.1. Determinations of pH were made colorimetrically with Clark and Lubs indicators corrected for salt error.

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[9] There is a corresponding reversal of the pH change when cells are replaced in normal sea water. In a very regular time course the original low pH is regained although somewhat more slowly than the rise to higher values. This slower exit may account for the apparent hysteresis in the recovery of P.D.

in entering the cell must leave a more acid region of sea water just outside the protoplasm; with the best of stirring this would probably extend the thickness of the cell wall, and therefore decrease the outside NH_3 concentration. These combined effects, more acid sea water outside, more alkaline sap inside, than are shown by the gross determinations, would greatly reduce the supposed gradients. The influence of such unstirred films on accumulation has been pointed out by Osterhout.[10]

Another factor which might make the pH rise less than expected would be an increase of (NH_4^+); this would cause (NH_3) to reach

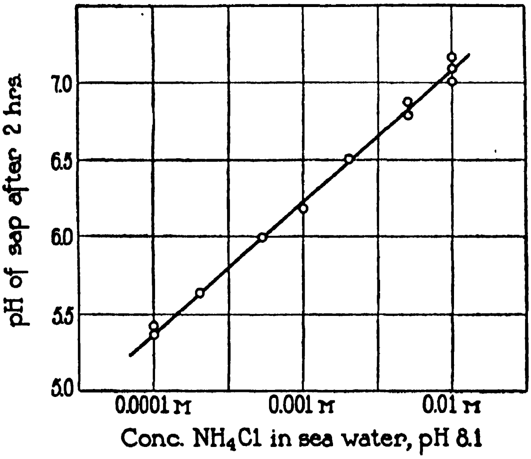

FIG. 9. pH of sap at "equilibrium" or steady values reached in Fig. 8 plotted against NH_4Cl concentrations in sea water (on logarithmic scale).

equilibrium at a lower pH, in accordance with equation (2). Presumably NH_3 would combine with acid when it entered the sap, and with a given concentration of acid, the amount of NH_4^+ formed could be calculated from equation (2) by the deviation of expected from observed pH, at equilibrium. Since there is already a large amount of ammonium salt in the sap of these cells, the relative amount of its increase is evidently not great, or there would be larger deviations than those found.

However, we are probably not dealing with equilibria, nor with concentration gradients alone. The continuous production of acid by the cell tends to keep down the internal pH, and this may be stimulated to an even higher rate by the entrance of ammonia. Such a compensation has been reported by Cooper and Osterhout[6] in the case of *Valonia*, where the pH of the sap eventually fell from its first high value, over longer periods in the presence of ammonia. In *Halicystis* there is evidence of an even prompter response of this sort, possibly accounting for the temporary lag of pH rise between 20 and 30 minutes exposure to ammonia, shown in Fig. 8; for the cusp following P.D. reversal (Figs. 2 to 4); and finally for the positive overshooting which occurs in the recovery from ammonia exposures (Fig. 2). All of these could be accounted for by a compensatory gush of acid production following the rise of pH produced by the entrance of NH_3. This would no doubt occur in the protoplasm rather than

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[10] Osterhout, W. J. V., *J. Gen. Physiol.*, 1932-33, **16**, 529.

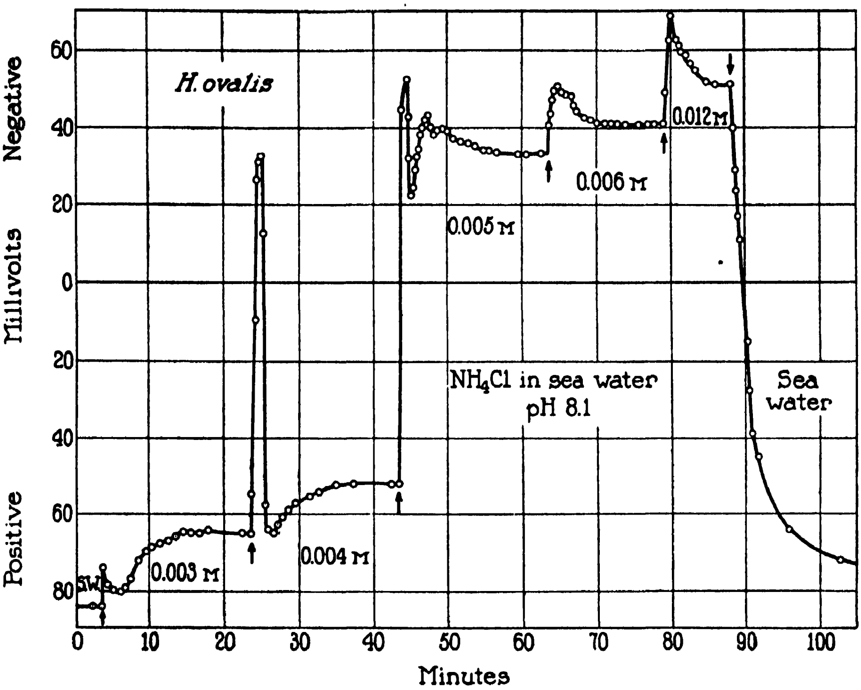

FIG. 10. Variation of P.D. in *Halicystis ovalis* showing extreme exaggeration of the cusp on reversal (with 0.004 M NH_4Cl in sea water at pH 8.1), causing only temporary reversal, followed by recovery of positivity. This may be due to a gush of acid production by the cell, compensating the rise of pH caused by entering NH_3. Arrows indicate change of solution.

in the sap, and it might be in the nature of glycolysis, rather than of increased respiration. An extreme case of its possible effect is shown in Fig. 10 (for *H. ovalis*) where some such influence caused complete recovery of P.D. after a brief reversal, a lasting reversal occurring only at the next higher NH_4Cl concentration. The almost invariably occurring cusp (*d* of Fig. 2) might be taken as an incomplete response of this sort, but insufficient to cause complete recovery.[11]

Some of these relations are of more interest from the viewpoint of salt accumulation than of potential difference. They are discussed at this length not so much for their bearing upon the pH and salt concentration in the sap, which is after all accessible both to analysis and to direct alteration *in vivo*, but rather as showing how any aqueous phase, separated from the sea water by a non-aqueous phase permeable to NH_3, might be expected to behave. The actual pH values of aqueous phases in the protoplasm we cannot directly determine, but if the sap can in any way be taken as representative, such pH will depend upon the original amount of ammonium salts and the buffer capacity of those phases when further ammonia enters. When the sap pH is 6.1, the pH of any given aqueous phase of the protoplasm may not be 6.1, probably is not. But we may be reasonably sure that it will bear some regular and probably linear relation to the NH_3 outside, and hence also to the NH_3 and to the pH of the sap.

Fig. 11, a combination of Figs. 5 and 9, summarizes at a glance the relation of the change in sap pH, which is proportional to log (NH_4Cl), or log (NH_3), in sea water; and the P.D., which passes through an abrupt inflection when the sap pH increases from 6.0 to 6.5.

We may now ask if in practice, such a change of pH could give rise to the S-shaped P.D. curve. The answer, based upon perfusing new solutions directly through the vacuole, is in the affirmative. When freshly extracted natural sap is made more alkaline, *e.g.* brought to pH 7 or 8 by the addition of a trace of NaOH, and then perfused into the vacuole of an impaled cell, practically the same results are obtained as when NH_4Cl above threshold concentrations is applied outside. The P.D. rapidly reverses from positive to negative, and stays reversed as long as the pH is kept this high. But since natural sap contains, as we have seen above, varying amounts of ammonium salt (often well above 0.001 M, the average outside threshold) NH_3 might have passed out from the sap into the protoplasm when the pH was raised, and produced its effects within the protoplasm exactly

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[11] The cusps might be interpreted as the somewhat similar ones caused by applying KCl to *Valonia* were interpreted by Damon:[5] as due to an advancing concentration boundary striking a surface of the protoplasm and then passing across it. Such an ammonium ion boundary might be formed within the protoplasm, due to the entrance of HN_3, but the absence of very large ammonium ion effects either on the outer or vacuolar surface of the protoplasm seems to rule out this explanation.

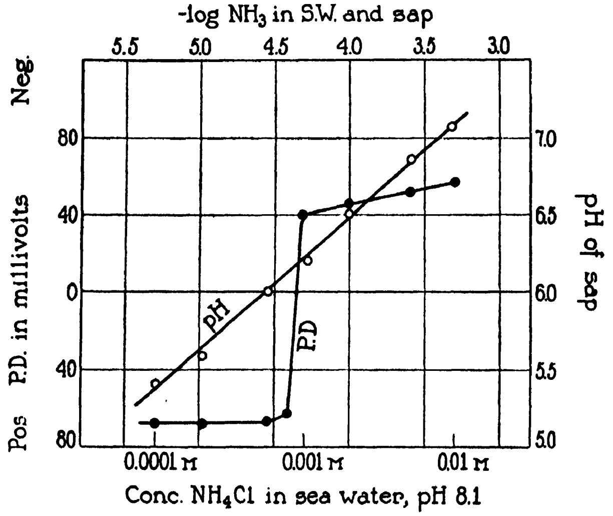

FIG. 11. Combined plot of the relations between concentration of NH_4Cl in sea water, log (NH_3) in sap and sea water, pH of sap, and P.D. across protoplasm of *Halicystis Osterhoutii.* The abrupt inflection of the P.D. curve is seen to be reflected in none of the other variables.

as if derived from the sea water. Fortunately it was eventually found possible to perfuse artificial sap and even sea water through the vacuoles. The P.D. remained positive and nearly normal as long as these were maintained at pH 5; and they could be circulated for an hour or more, in order to make sure that most of the original sap had been washed out, with its ammonium salt. Then when more alkaline artificial sap or sea water was perfused, the typical reversal of P.D. still occurred. Furthermore, the pH at which the reversal occurred coincided remarkably well with that produced inside by the application of threshold concentrations of NH_4Cl outside; namely, between pH 6.0 and 6.5. Thus in one case the P.D. stayed normally positive with perfusion of sea water buffered at pH 6.0, but reversed with continued perfusion at pH 6.2. The ammonia effects seem therefore to be accounted for, qualitatively at least, by the pH change occurring in the sap. A complete description of these perfusion experiments, and a discussion of the possible systems upon which such a change of pH might operate to reverse the potential, will appear in a forthcoming paper.

SUMMARY

The nature and origin of the large "protoplasmic" potential in *Halicystis* must be studied by altering conditions, not only in external solutions, but in the sap and the protoplasm itself. Such interior alteration caused by the penetration of ammonia is described. Concentrations of NH_4Cl in the sea water were varied from 0.00001 M to above 0.01 M. At pH 8.1 there is little effect below 0.0005 M NH_4Cl. At about 0.001 M a sudden reversal of the potential difference across the protoplasm occurs, from about 68 mv. outside positive to 30 to 40 mv. outside negative. At this threshold value the time curve is characteristically S-shaped, with a slow beginning, a rapid reversal, and then an irregularly wavering negative value. There are characteristic cusps at the first application of the NH_4Cl, also immediately after the reversal.

The application of higher NH_4Cl concentrations causes a more rapid reversal, and also a somewhat higher negative value. Conversely the reduction of NH_4Cl concentrations causes recovery of the normal positive potential, but the threshold for recovery is at a lower concentration than for the original reversal. A temporary overshooting or increase of the positive potential usually occurs on recovery. The reversals may be repeated many times on the same cell without injury.

The plot of P.D. against the log of ammonium ion concentration is not the straight line characteristic of ionic concentration effects, but has a break of 100 mv. or more at the threshold value. Further evidence that the potential is not greatly influenced by ammonium ions is obtained by altering the pH of the sea water. At pH 5, no

reversal occurs with 0.1 M NH_4Cl, while at pH 10.3, the NH_4Cl threshold is 0.0001 M or less. This indicates that the reversal is due to undissociated ammonia.

The penetration of NH_3 into the cells increases both the internal ammonia and the pH. The actual concentration of ammonium salt in the sap is again shown to have little effect on the P.D. The pH is therefore the governing factor. *But assuming that NH_3 enters the cells until it is in equilibrium between sap and sea water, no sudden* break of pH should occur, pH being instead directly proportional to log NH_3 for any constant (NH_4) concentration. Experimentally, a linear relation is found between the pH of the sap and the log NH_3 in sea water. The sudden change of P.D. must therefore be ascribed to some system in the cell upon which the pH change operates. The pH value of the sap at the NH_3 threshold is between 6.0 and 6.5 which corresponds well with the pH value found to cause reversal of P.D. by direct perfusion of solutions in the vacuole.

THE INACTIVATION OF BACTERIOPHAGE BY MERCURY BICHLORIDE; THE REACTIVATION OF BICHLORIDE-INACTIVATED PHAGE

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There are at the present time two schools of thought with reference to the nature of bacteriophage. One group, championed chiefly by d'Herelle and Burnet, considers phage to consist of living "corpuscles," while the other group views the lytic principle as partaking of all the major characteristics of an enzyme.

A study of the kinetics of the bacterium-bacteriophage reaction by Krueger and Northrop (1, 2), together with additional data reported by Krueger concerning first, the sorption of bacteriophage by living and by dead bacteria (3) and second, the heat inactivation of bacteriophage (4), gave indirect information tending to support the enzyme concept of phage. During further experimental work one of us (A. P. K.) on several occasions noted apparent significant reversals of phage inactivation. These data were interpreted as indicating either the independent multiplication of residual active phage in the absence of growing bacteria—a phenomenon not hitherto proven to occur—or the reactivation of inactivated phage, a reaction common to many enzymes. The present paper details experiments undertaken to investigate the inactivation of antistaphylococcus bacteriophage by $HgCl_2$ and the reversal of the process.

Methods

An antistaphylococcus phage and a single strain of *S. aureus* described in previous papers were used (1–6). The medium was beef infusion broth containing 1 per cent peptone, 0.5 per cent sodium chloride, adjusted to pH 7.6. All phage titrations were performed according to the method described by Krueger (5, 6).

The titration procedure rests upon the observation that the time of lysis in a standard bacterial suspension under standard conditions is a function of $[P]_0$, the initial concentration of phage. Since the initiation of lysis depends upon the attainment of a critical P/B ratio and since also $dP/dt \propto C \times dB/dt$, it is clear that any possible inhibition of bacterial growth by $HgCl_2$ in the titration set-up must be ruled out. As a means of determining the optimal dilutions for phage titration the growth of the bacterium in broth to which had been added various amounts of $HgCl_2$ was followed. It was found that a concentration of 1:10,000,000 of $HgCl_2$ exerted no measurable inhibition on the growth of the organism. Consequently throughout the experiments the phage determinations were carried out with dilutions containing 1:10,000,000 or less of $HgCl_2$.

1. The Inactivation of Phage by $HgCl_2$.—5 ml. of 1:5,000 $HgCl_2$ were added to an equal volume of standard phage containing 1×10^{10} activity units per ml. The mixture was maintained at 22°C. and samples for phage titration were removed at intervals; they were at once diluted 1:1,000 with broth.

2. The Reversal of Phage Inactivation by $HgCl_2$.—5 ml. of standard phage were mixed with 5 ml. of 1:5,000 $HgCl_2$. The mixture was kept at 22°C. for 0.5 hour at which time samples were secured for titration of the residual active phage and a 1 ml. sample was taken for the reversal procedure. This last was mixed immediately with 1 ml. of a saturated solution of H_2S in water and was set aside for 12 minutes. It was then centrifuged at high speed to remove the fine precipitate of HgS. The supernatant was pipetted off, thoroughly aerated until free from H_2S, and diluted for the phage titration.

Controls consisted of: (*a*) phage diluted with 1:10,000,000 $HgCl_2$ to check any possible effect of this concentration on the titration results, and (*b*) phage exposed to the H_2S and aerated as was done with the reversed fraction.

EXPERIMENTAL RESULTS

1. Inactivation of Phage.—Fig. 1 is a composite plot of the results obtained in a series of experiments on the inactivation of phage by $HgCl_2$ at 22°C. The inactivation proceeds logarithmically with time and is therefore a pseudomonomolecular reaction with one reactant ($HgCl_2$) greatly in excess of the other, so that its concentration remains practically constant throughout the experiment.

The reaction may then be expressed as $dP/dt = k\,[HgCl_2]\,[P_o - P_t]$ where

P = bacteriophage in activity units,
P_o = initial phage concentration,
P_t = phage inactivated in time t,
and $[HgCl_2]$ = concentration of bichloride of mercury.

This, on integration, gives $k = 1/t\,[HgCl_2] \cdot \ln P_o/[P_o - P_t]$. It will be noted that in the plot of experimental data the curve does not

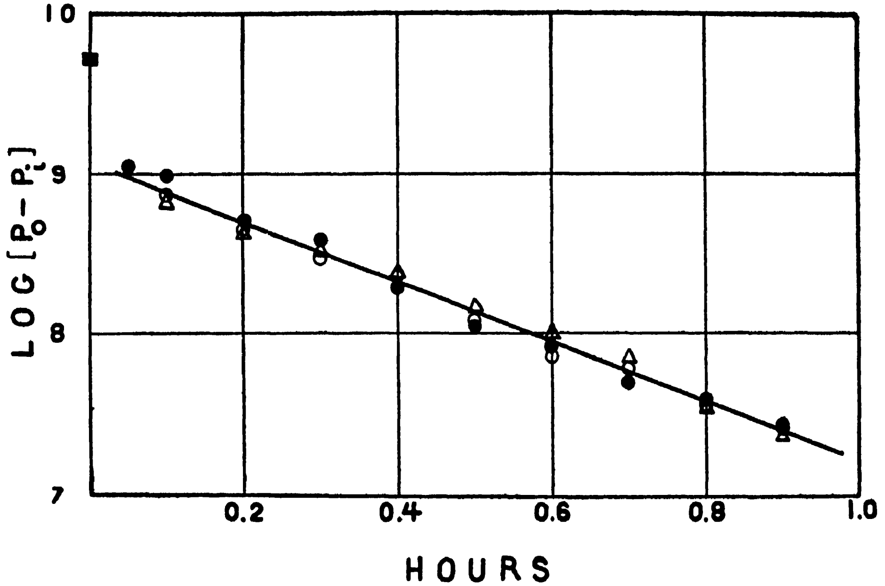

FIG. 1. Composite plot of logarithms of residual active phage units against time during $HgCl_2$ inactivation of phage at pH 7.6 and 22°C. (three experiments). ■ represents the common origin at $t = 0$ for all three initial phage concentrations.

TABLE I

Summary of Four Separate Experiments in All of Which Phage Was Inactivated with $HgCl_2$

The initial [P] was 5 × 10^9. In three cases reactivation following removal of Hg^{++} with H_2S restored the [P] to its original titre (100 per cent reversal).

A	B	C	D	E	
Inactivated phage 1:10,000 $HgCl_2$ 0.5 hour	Reactivated phage As A. Followed by H_2S. Centrifuged. Aerated till H_2S-free	Control 1 × 10^{10} phage units + H_2S. Aerated till H_2S-free	Control 1 × 10^{10} phage units titrated in presence of 1:10,000,000 $HgCl_2$	Original phage present after	
				Inactivation	Reactivation
				per cent	*per cent*
(1) 2.2 × 10^8	5.0 × 10^9	9.0 × 10^9	Not included in this experiment	4.4	100
(2) 1.3 × 10^8	2.1 × 10^9	8.9 × 10^9	Not included in this experiment	2.6	42
(3) 6.3 × 10^8	5.0 × 10^9	1.0 × 10^{10}	1.0 × 10^{10}	12.6	100
(4) 2.5 × 10^8	5.0 × 10^9	1.0 × 10^{10}	1.0 × 10^{10}	5.0	100

originate in the actual $[P]_0$ used. We have observed in the broth suspension of phage a faint, fine precipitate appearing at once upon the addition of $HgCl_2$. This precipitate develops in an identical fashion in plain broth and it seems likely that a certain amount of phage is carried down with it accounting for the immediate precipitous drop in $[P]$.

2. *Reactivation of Inactivated Phage.*—Table I is a summary of four experiments in which standard phage was subjected to the action of $HgCl_2$ for 0.5 hour. The Hg^{++} ion was then removed with H_2S. In three of the four experiments reactivation was complete; that is, the final concentration of active phage was 100 per cent of that originally present before inactivation. In the fourth experiment the final phage titre was 50 per cent of $[P]_0$.

DISCUSSION

The data given above show first that the inactivation of phage by $HgCl_2$ follows the course of a pseudomonomolecular reaction, and secondly, that when the mercury ion is precipitated from the mixture an increase in $[P]$ occurs. The increase in phage concentration after removal of the Hg ions with H_2S may be explained in several ways.

First, it is possible that in the presence of H_2S, phage actually multiplies. Such is probably not the case, however, for one of the few points concerning phage upon which agreement is general, is that growth of a susceptible organism conditions phage multiplication. There are no recorded critical experiments in the literature indicating that $[P]$ can be increased by any other agency. Further, our controls exposed to H_2S and aerated, as in the reversal series, exhibit, if anything, a slight decrease in $[P]$.

Second, enough H_2S may be left behind after aeration to exert a stimulating effect on bacterial growth, consequently shortening the time of lysis in the titration set-up and thereby effecting an apparent increase in the initial $[P]$. Here again, the H_2S controls rule out such an occurrence for they too would exhibit this effect.

One alternative explanation remains; namely, that $HgCl_2$ inactivation of phage is actually reversed by precipitation of Hg^{++} ions with H_2S. This fits the observed data.

CONCLUSIONS

1. The inactivation of antistaphylococcus bacteriophage suspended in infusion broth at pH 7.6 and 22°C. by $HgCl_2$ proceeds according to the equation $dP/dt = k\,[HgCl_2]\,[P_o - P_i]$ over the range studied.

2. This inactivation can be reversed by precipitation of Hg^{++} with H_2S. In the present experiments the inactivation was carried out until only some 5 per cent of the initial phage remained active. After reactivation the $[P]$ had increased to 100 per cent of the initial $[P]$.

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THE EFFECT OF ULTRAVIOLET LIGHT ON PHOTOSYNTHESIS

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I

In studying the killing of small organisms by x-rays, α-rays, and ultraviolet light, a number of investigators (Crowther, etc. (1)) have found that the results could be most simply explained by the assumption that one quantum or one α-particle caused death by hitting a cell. These results are of interest for two reasons. First, quantum mechanisms may possibly exist in living material. Second, the method of studying small structures by their interaction with photons is not subject to the limitations imposed on the microscopic method by the wave nature of light. (An example of this is DuMond's recent investigation—by the Compton effect—upon the magnitude and distribution of velocities of electrons inside the atoms of solid metals (DuMond (2)).)

This paper has to do with the effect of the ultraviolet radiation (Hg 2537 Å) on the green alga *Chlorella pyrenoidosa.* However, death will not be used as an end-point because it might result from a number of different causes and is difficult to define and to test for. Instead, attention will be concentrated on some function of the cell—respiration, fermentation, or photosynthesis—on the assumption that the mechanism of that function is more uniform in its sensitivity to the radiation than are the cells themselves. The effect of the radiation on this function will be studied manometrically by the method described by Warburg (3) and Emerson (4). This method allows the use of a far larger number of individuals than can be used when counts must be made. One experiment involves 150 million cells, thus reducing the rôle of statistical variation to a minimum.

II

The device for determining the number of quanta involved is as follows:

One Quantum Hit

If we have N individuals of Type A, one quantum can change an A to a B.

$$A + h\nu \rightarrow B;$$

the rate of destruction of A is given by

$$-\frac{dN}{dt} = NQP \tag{I}$$

where Q is the rate of absorption of quanta by one A.

$$Q = \frac{I\mu V}{h\nu}$$

when

I = light intensity

μ = absorption coefficient

V = volume of A

and P is the probability of an absorbed quanta effecting the change A to B. (P admits the possibility of an absorbed quantum not making a change in A.)

The solution of equation (I) is—

$$N = N_o e^{-QPt}, \tag{II}$$

N_o = number of A present before irradiation;

t = time of irradiation.

Equation (II) gives

$$\ln\left(\frac{N}{N_o}\right) = -QPt \tag{III}$$

Plotting $\ln\left(\frac{N}{N_o}\right)$ (*survival ratio*) against time gives the graph shown in Fig. 1. The value of the slope will be $-QP$.

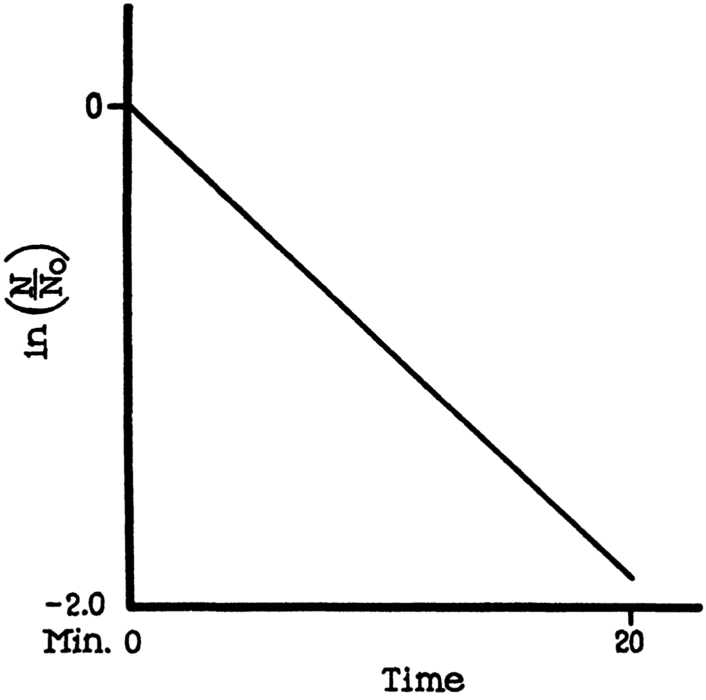

FIG. 1

Two Quanta Hit

If it takes two quanta to effect the change of A to B, we obtain:

$$A + h\nu \rightarrow A'$$

where A′ cannot be distinguished by the experiment from A.

$$A' + h\nu \rightarrow B$$

B = the "killed" or inactive form of A.
Q = the rate at which quanta are absorbed by one A or A′.
P = the probability of a hit being effective. (It should be stated that P might be different for A and for A′.)
N_0 = number of units of A before irradiating.
N = number of units of (A + A′) at any time.
t = time of irradiation.
S = number of units of A at any time.

From equation (II) we have

$$S = N_0 e^{-QPt}.$$

$-\frac{dN}{dt}$ will be proportional to the number of A's present, that is, to $(N - S)$:

$$-\frac{dN}{dt} = (N - S)QP = QP(N - N_0 e^{-QPt}).$$

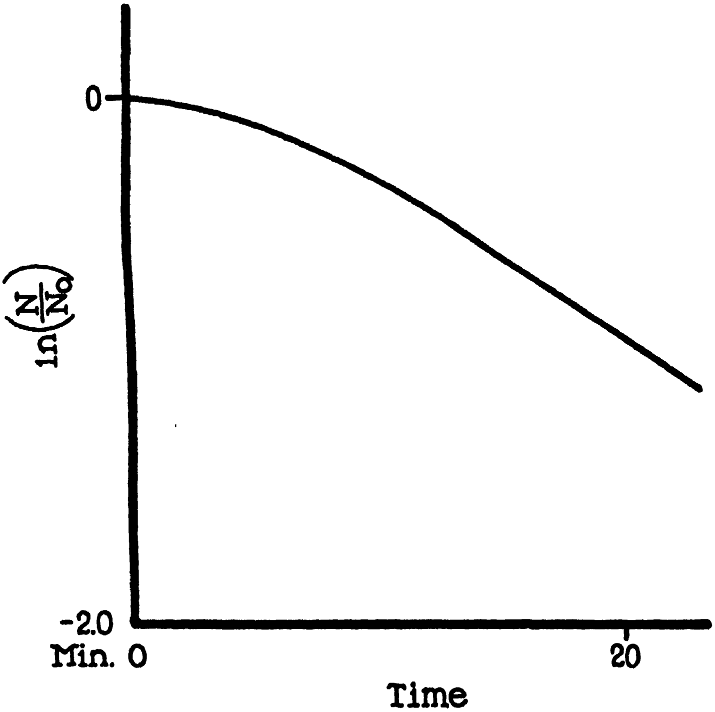

FIG. 2

The solution is

$$N = e^{-QPt}(QPN_0t + C). \tag{IV}$$

$N = N_0$ when $t =$ zero, so that the constant $C = N_0$ and

$$\frac{N}{N_0} = e^{-QPt}(1 + QPt);$$

taking the log of each side we have

$$\ln\left(\frac{N}{N_0}\right) = -QPt + \ln(1 + QPt). \qquad \text{(V)}$$

Plotting ln (*survival ratio*) against time, we have the curve given in Fig. 2. A comparison of Figs. 1 and 2 shows the possibility of determining the number of quanta involved in the change from the shape of the curve for ln (*survival ratio*) against time. As the number of quanta involved increases, the curves shift progressively to the right (Curie (1)).

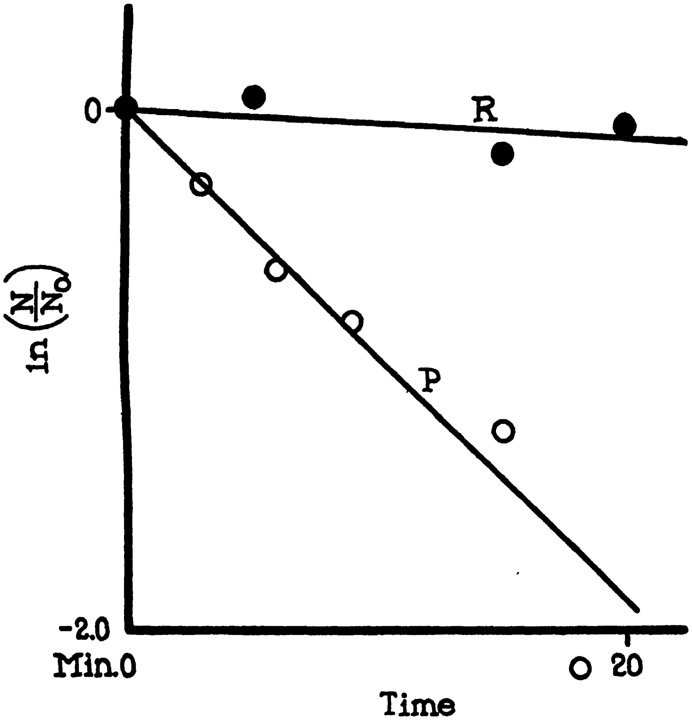

FIG. 3. *R* is the curve for respiration. Shows no effect. *P* is the curve for photosynthesis. It shows one quantum-to-kill type of effect.

III

For the experiments 5 mm.^{3} (150 million) of *Chlorella pyrenoidosa* cells were suspended in 1.5 cc. of Warburg's carbonate buffer (Mixture IX), (Warburg (3)), in a small quartz Warburg vessel. Photosynthesis and respiration were measured as a function of the time of irradiation. The cells were rayed in the vessel with the light from a quartz mercury tube. It is possible by electrodeless discharge in mercury vapor induced by a radio frequency coil ($\lambda = 3$M.) to obtain light from 90 to 96 per cent monochromatic for the 2537 Å line.

When the ln (*survival ratio*) for respiration and for photosynthesis are plotted against the time of irradiation we obtain the kind of result shown in Fig. 3.

P, the probability of a hit being effective, may be determined from the following experiment. 5 mm.^{3} of cells were used as before. The energy output of the ultraviolet tube was measured by Dr. F. L. Gates and found to be 37.4 ergs per mm.^{2} per second at the point where the quartz vessel was placed. The area exposed was 160 mm.^{2} Measurements with a Weston cell (quartz window) showed that 52 per cent of the incident light was absorbed by the *Chlorella* suspension. This means that the cells absorbed 3.1×10^3 ergs per second. The energy of one quantum of 2537 Å wave length is 7.7×10^{-12} ergs. By dividing this number into the energy absorbed we obtain 4×10^{14} as the number of quanta absorbed per second.

TABLE I

The Effect of Ultraviolet Radiation on Photosynthesis

	Rate	ln rate
	Δh mm. for 5 min. period	
Before irradiation	25.4	3.2348
After 5 min. ultraviolet irradiation	15.2	2.7213

For 5 minutes $\ln\left(\frac{N}{N_0}\right) = -0.5135$ or the slope $= \frac{-0.5135}{300} = -1.7 \times 10^{-3}$ second^{-1}.

The numerical value of *P* depends upon what element we assume to be destroyed by the ultraviolet light. Table II gives the calculations for three possible assumptions: first, individual *Chlorella* cells; second, photosynthetic units; third, chlorophyll molecules.

According to hemocytometer counts there are 30 million *Chlorella* cells per mm.^{3} of the cell material.

Defining the number of photosynthetic units as the number of carbon dioxide molecules reduced per flash when the flashes are so far apart that they do not interfere with one another, and when the flashes are bright enough to produce light saturation—then the number of photosynthetic units is

$$\frac{13.2 \times 0.185 \times 6.06 \times 10^{23}}{5 \times 60 \times 15 \times 1000 \times 22{,}400} = 1.46 \times 10^{13}$$

where

13.2 mm. = the change of the manometer pressure in 5 minutes (determined previous to the irradiation by ultraviolet light).

0.185 = the vessel constant

15 = the number of flashes per second.

TABLE II

Three Possible Values of P

	N	Q	P
Element assumed to be hit by ultraviolet light	Original No. present in vessel	Average No. of quanta absorbed per unit per second	Probability $\frac{1.7 \times 10^{-3}}{Q}$
Chlorella cell	150×10^{6}	2.7×10^{6}	6.3×10^{-10}
Photosynthetic unit	1.46×10^{13}	2.7×10	6.3×10^{-5}
Chlorophyll molecule	3.6×10^{16}	1.1×10^{-2}	1.5×10^{-1}

The number of chlorophyll molecules is equal to the number of photosynthetic units multiplied by 2480 (Emerson and Arnold (5)). Although the relative probabilities are correct, the absolute magnitudes are subject to an error perhaps as large as 500 per cent. It has not been possible as yet to grow two cultures giving identical slopes. Furthermore, the light intensity has not been corrected for reflection, or for absorption by different parts of the cell. A new absorption cell is now being made with which the absorbed energy can be determined with greater precision.

The value 1.5×10^{-1} for P suggests at once that it is the chlorophyll molecule which is affected by ultraviolet light. To test this assumption arrangements were made with Professor J. B. Conant and Dr.

Dietz to examine the chlorophyll chemically. A culture of *Chlorella pyrenoidosa* was irradiated with ultraviolet light until manometric tests showed that the photosynthesis had been reduced to less than 10 per cent of its original rate. The culture was then given to Dr. Dietz who made extracts within less than 1 hour from the time of irradiation. Previous measurements had shown that the damage to photosynthesis by ultraviolet light lasts for at least 7 hours. The following is Dr. Dietz' report:

> "The suspension of *Chlorella* was centrifuged, washed once with distilled water and the chlorophyll was extracted by grinding with sand in the presence of acetone. Ether was added to the acetone solution and the acetone removed by washing carefully with water.
>
> Suitable tests showed that the chlorophyll was unchanged chemically. A prolonged yellow phase color was obtained on shaking the ether solution with methyl alcoholic potassium hydroxide, hence no allomerization had taken place. Neither 0.01 N potassium hydroxide nor 22 per cent hydrochloric acid extracted any of the pigment, hence the phytyl group had not been removed. A hot quick saponification carried out according to the Willstätter procedure followed by methylation and acid fractionation indicated that chlorin *e* and rhodin *g* esters were the sole products and were formed in the normal 3 to 1 ratio. This showed that no oxidation of the chlorophyll or alteration of the ratio of the *a* and *b* components had taken place."

IV

CONCLUSIONS

The fact that the chlorophyll appears to remain unchanged chemically allows two conclusions,—either that there is a change so subtle that it escapes detection, or that the ultraviolet light destroys a substance other than chlorophyll. If the first proves true, then a mechanism like that suggested by Conant, (Conant, Dietz, and Kamerling (6)) may be used to explain the high ratio between chlorophyll content and photosynthesis per flash (Emerson and Arnold (5)). If the second is the correct conclusion, then the hypothetical substance must be proportional to, and a very small fraction of, the chlorophyll content of the cell. The probability calculated for the photosynthetic unit fits this conclusion—because most of the absorbed quanta would be taken up by the chlorophyll which is present in a much higher concentration than is the hypothetical substance. However, Warburg's high light efficiency for *Chlorella* is difficult to understand from this point of view. We would expect the chlorophyll to act as an internal screen.

It is probable that when the mechanism of the photosynthesis of green plants is finally described, it will be found that the study of the quantum relationships of both the visible and ultraviolet light have played an important part.

The writer is much indebted to Dr. E. M. Dietz, Professor J. B. Conant, Professor W. J. Crozier, and Dr. F. L. Gates for help and advice.

V

SUMMARY

1. An unidentified unit in the mechanism of the photosynthesis of *Chlorella pyrenoidosa* is rendered inactive by the absorption of one quantum of ultraviolet light (2537 Å wave length).

2. The same irradiation has no effect on the normal respiration of *Chlorella pyrenoidosa*. Experiments have not yet been made on the respiration inhibitable by HCN.

3. No chemical change was detected in the chlorophyll extracted from irradiated cells.

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THE ORDER OF THE BLACKMAN REACTION IN PHOTOSYNTHESIS

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I

Previous studies (Emerson and Arnold (1, 2)) have shown that the photosynthesis of *Chlorella pyrenoidosa* involves a photochemical reaction of the first order not appreciably affected by temperature, and a dark reaction (the Blackman reaction) dependent on temperature. This paper will attempt to prove that the Blackman reaction is of the first order. The argument will be based on the effect of ultraviolet light on photosynthesis.

II

ARGUMENT

It has been shown (Arnold (3)) that ultraviolet light (2537 Å) destroys the photosynthetic mechanism in *Chlorella pyrenoidosa.* There are two ways to measure this destruction of photosynthesis: first, by using bright flashes of light spaced so far apart that the measurements are independent of the Blackman reaction; second, by very bright and continuous light in which the Blackman reaction will be the pace setter. The order of the Blackman reaction can be determined from the relationships between the survival ratios for the two methods of measuring photosynthesis. The survival ratio is defined as the rate of photosynthesis after irradiation by ultraviolet light, divided by the original rate of photosynthesis.

Let

N = the number of units ready to undergo the photochemical reaction.

n = the number of units ready to undergo the Blackman reaction.

x = the order of the Blackman reaction, 1, 2, 3, ...

Since the process as a whole is thought to be a cyclic one,

$$N + \frac{n}{x} = K.$$

where K_o is a constant for the cells, proportional to their chlorophyll content. After irradiation by ultraviolet light K_o is replaced by a smaller number K. This substitution represents the damaging of the photosynthetic mechanism by ultraviolet light.

Derivation of the Survival Ratio for Flashing Light

The rate of the photochemical reaction is (Emerson and Arnold (2)),

$$-\frac{dN}{dt} = A\,I\,N$$

where

A = a reaction constant nearly independent of temperature.

I = the light intensity.

(The Blackman reaction may be neglected because it is very slow compared with the time of the light flash, 10^{-5} sec. (Emerson and Arnold (2)).) The solution of the above equation after substituting K_o for the integration constant is

$$N = K_o e^{-A\int I dt}.$$

When the $\int I\,dt$ is evaluated over one flash of light

$$N_1 = K_o e^{-A\int I dt},$$

where N_1 = number of units unchanged by the light. The photosynthesis M_o effected by one flash will be given by

$$M_o = K_o - N_1 = K_o(1 - e^{-A\int I dt})$$

Similarly for M the photosynthesis effected by one flash after the ultraviolet treatment, we have

$$M = K(1 - e^{-A\int I dt}).$$

The gas exchange measured in a 5 minute period is proportional to M_o and M, because there will be the same number of flashes in each 5 minute interval. That is, there will be 5 × 60 × 15 (for fifteen flashes per second).

Then

$$\left.\begin{array}{r}\textit{The survival ratio}\\ \textit{for flashing light}\end{array}\right\} = \frac{M}{M_o} = \frac{K(1 - e^{-A\int I dt})}{K_o(1 - e^{-A\int I dt})} = \frac{K}{K_o}$$

Derivation of Survival Ratio for High Intensity Continuous Light

R_o, the rate of the Blackman reaction, is

$$R_o = B n^x$$

where B is the reaction constant dependent on temperature. If the light intensity is high enough to effect light saturation for the plants, then

$$N = 0,$$

so that from the equation

$$N + \frac{n}{x} = K_o$$

we obtain

$$n = xK_o$$

Substituting this value for n in the equation for rate we have,

$$R_o = B(xK_o)^x$$

In order to find the rate after ultraviolet irradiation the assumption is made that ultraviolet light does not affect the reaction constant B,

$$R = B(xK)^x.$$

These rates are the same as the experimentally determined rate for the photosynthetic process as a whole.
Then,

$$\left.\begin{array}{r}\textit{The survival ratio}\\ \textit{for high intensity}\\ \textit{continuous light}\end{array}\right\} = \frac{R}{R_o} = \frac{B(xK)^x}{B(xK_o)^x} = \left(\frac{K}{K_o}\right)^x$$

But this is the survival ratio for flashing light raised to the power x, the order of the Blackman reaction.

III

EXPERIMENTAL

5 mm.^{3} of *Chlorella pyrenoidosa* cells were washed and then suspended in a carbonate buffer solution (85 parts of 0.1M potassium bicarbonate

and 15 parts of 0.1M potassium carbonate) in a Warburg vessel. Photosynthesis was measured by reading the manometers every 5 minutes, first when the cells were exposed to very high continuous light, and second when the cells were illuminated by 10^{-5} second flashes, 15 per second. The cells were then irradiated for 5 minutes by ultraviolet light, more than 90 per cent monochromatic for the 2537 Å line of mercury. Photosynthesis was again measured and survival ratios determined. The results appear in Table I.

According to the argument in Section II, the power to which the survival ratio in flashing light must be raised to give the survival ratio in continuous light corresponds to the order of the Blackman reaction.

TABLE I

Survival Ratios for High Intensity Continuous Light and for Bright Flashing Light

	Δ h mm. for 5 min. period before irradiation		Δ h mm. for 5 min. period after 5 min. of ultraviolet irradiation		Survival ratios	
Experiment	Continuous light	Flashing light	Continuous light	Flashing light	Continuous light	Flashing light
1	25.4	13.2	15.2	7.8	0.60	0.59
2	21.3	11.3	11.5	6.2	0.54	0.55

The tabulated data in the light of this argument are only consistent with a Blackman reaction of the first order.

IV

Since one molecule of glucose contains six carbon atoms, and since it takes at least three quanta of visible light to obtain enough energy to reduce one molecule of carbon dioxide, it is hard to conceive of the process of photosynthesis consisting of first order reactions. Perhaps photosynthesis may be pictured as in Fig. 1. Let the arrow A represent the manufacture of sugar, the arrow P the photochemical process, the arrow B the Blackman reaction. P and B make up a turning wheel driven by the light energy. The rotation of this wheel moves the process as a whole. If some mechanism like that suggested by Conant (Conant, Dietz, and Kamerling (4)) proves to be applicable, then the constant K_0 would represent the maximum amount of dehydrochlorophyll that can be present at one time. Whether it will be possible to explain by Professor Conant's scheme the ratio 2,500 between chlorophyll content and carbon dioxide reduced per flash remains to be seen.

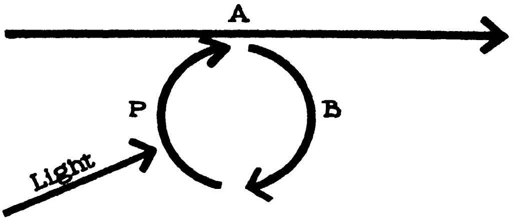

Fig. 1

V

SUMMARY

On the assumption that photosynthesis is a cyclic process and that irradiation by ultraviolet light does not change the reaction constants, the Blackman reaction is shown to be of first order.

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THE ESTIMATION OF TRYPSIN WITH HEMOGLOBIN

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(Accepted for publication, August 10, 1933)

The method for the estimation of trypsin described in this paper is essentially the same as our method for the estimation of pepsin (Anson and Mirsky, 1932). Trypsin is allowed to digest denatured hemoglobin in a slightly alkaline phosphate solution. Precipitation of the denatured hemoglobin by the phosphate or by salt added with the enzyme is prevented by urea. The undigested hemoglobin is precipitated with trichloracetic acid. The amount of digested hemoglobin not precipitated, which is a measure of the amount of trypsin used, is estimated by the blue color which the tyrosine and the tryptophane in the digested hemoglobin give with the phenol reagent. Cysteine and heme (even heme whose iron is in the ferric state) also can reduce the phenol reagent. But there is very little cysteine in hemoglobin and all the heme is precipitated with trichloracetic acid.

The procedure has several advantages. Many estimations can be made in a short time; the results are entirely reproducible; the hemoglobin solution keeps for at least a month without change; and the rate of digestion is not sensitive to considerable amounts of acid, alkali, urea or glycerol added with the enzyme.

The procedure has two disadvantages. In the first place, the hemoglobin solution cannot be used for the estimation of the active native trypsin in a mixture of active native and inactive denatured trypsins because inactive denatured trypsin changes into active native trypsin in the hemoglobin solution just as it does in all the protein solutions which have hitherto been used for the estimation of trypsin. This change can be prevented by making the hemoglobin solution more alkaline as is described in a following paper. In the second place, the activity of a crude pancreatic extract is higher when measured by the digestion of hemoglobin than when measured by the change in the viscosity of gelatin. The reasons for this are being studied. In the experiments with purified trypsin so far carried out the two methods yield the same results.

Commercial dried proteins can be used instead of hemoglobin prepared in the laboratory. They are of dubious reproducibility and they contain considerable and variable amounts of color-producing substances not precipitated by trichloracetic acid.

When gelatin, casein or any other non-reproducible protein substrate is used for the estimation of trypsin by any method, the procedure can be calibrated by means of a solution of purified trypsin whose activity has been measured by the hemoglobin method which yields reproducible results. The calibration curve states the extents to which a particular sample of protein is digested under given conditions by different known amounts of trypsin. A sufficiently purified trypsin can be prepared from commercial trypsin in a few minutes by a modification of the Northrop-Kunitz procedure (1932) which avoids several filtrations. A solution of this partially purified trypsin which digests hemoglobin at the same rate as a solution of crystalline trypsin also has the same effect on the viscosity of gelatin as does the crystalline trypsin.

The Procedure.—1 ml. of enzyme solution is added to 5 ml. of the hemoglobin solution to be described later. The 175 × 20 mm. test-tube containing the 6 ml. of digestion mixture is whirled and placed in a water bath at 25°C. After 5 minutes 10 ml. of 5 per cent trichloracetic acid are poured in from another test-tube, the suspension is poured back and forth, allowed to stand 5 minutes and filtered. To 5 ml. of filtrate are added 10 ml. of 0.50 N sodium hydroxide and 3 ml. of the phenol reagent of Folin and Ciocalteau (1927) diluted three times (*cf.* Wu, 1922, and Greenberg, 1929). The reagent is added drop by drop with stirring and is always added in the same way. After 1 to 10 minutes the blue color is read against the color developed from 0.00083 milliequivalents (0.15 mg.) of tyrosine dissolved in 5 ml. of 0.2 N hydrochloric acid.

If the trichloracetic acid suspension is filtered immediately instead of after 5 minutes the first half of the filtrate contains some undigested hemoglobin in fine suspension and this first portion must accordingly be rejected or refiltered. Centrifugation can be used instead of filtration without any difference in results.

Preparation of Tyrosine Standard.—The tyrosine is thrice crystallized and its concentration is estimated by Kjeldahl (100 mg. tyrosine = 7.74 mg. nitrogen).

It is stored at room temperature in 0.2 N hydrochloric acid containing 0.5 per cent formaldehyde. Some preservative is needed to prevent the destruction of tyrosine by mould even in the cold. Formaldehyde does not affect the color value of tyrosine.

Copper sulfate solution or a blue glass inserted in the plunger of the colorimeter can be used as a standard instead of the blue solution obtained from tyrosine. Although these standards do not match the tyrosine blue in white light they do match it in the fairly monochromatic red light transmitted by the Corning Glass Filter No. 241.

Rubber, even after being boiled with alkali, contains reducing substances which can be extracted by the reagents so all contacts with rubber should be avoided.

Preparation of the Hemoglobin Solution.—Defibrinated bovine blood is centrifuged, the serum and white corpuscles are siphoned off and the red corpuscles are washed once with an equal volume of 0.9 per cent sodium chloride solution.[1] Water is added to give a solution containing in 100 ml. 10.5 gm. hemoglobin or 1.86 gm. nitrogen. This solution is stored frozen in paraffined paper ice-cream containers.

To denature the hemoglobin and to remove substances not precipitated with trichloracetic acid which give a color with the phenol reagent, one proceeds as follows. A mixture of 220 ml. 10.5 per cent hemoglobin and 11 ml. 1 N sodium hydroxide is brought to 50–60°C. and is added to 1300 ml. of water previously brought to 100°C. There is then added with mechanical stirring 26 ml. of a solution 5 M in respect to sodium chloride and 0.5 M in respect to KH_2PO_4. The resulting suspension is filtered on a folded paper, the precipitate is washed with water, transferred to a beaker, weighed and enough water added to make the weight 400 gm. 400 gm. of urea are then stirred up with the precipitate and 160 ml. of 1 N sodium hydroxide are added. After solution of the protein and the urea 200 ml. of 1 M KH_2PO_4 plus 240 ml. water are added. The solution is stored in the cold with toluol as a preservative. The solution is the same as would be obtained by adding 40 gm. of urea to 100 gm. of a solution which contains 2.2 gm. denatured hemoglobin (about 5 per cent of the protein is lost) and the equivalents of 100 ml. of 0.2 M KH_2PO_4 and 80 ml. of 0.2 M sodium hydroxide.

Preparation of Solutions of Commercial Dried Proteins.—25 gm. of edestin (La Roche) hemoglobin (Eimer and Amend) or casein (after Hammarsten) are mixed with 400 gm. urea. This mixing facilitates the solution of the protein. In the case of casein and edestin, the protein and urea are simply put together in a flask which is whirled. In the case of hemoglobin the protein and urea are ground together in a mortar. 240 ml. water and 160 ml. 1 N sodium hydroxide are added to the urea-protein mixture and the solution is brought to room temperature. After the protein is dissolved (and denatured) 200 ml. 1 M KH_2PO_4 and 375 ml. water are added. Eimer and Amend's hemoglobin dissolves more readily than other commercial hemoglobins we have tried. Although it is labelled pure it is contaminated with other proteins. Of the three proteins, edestin is the most rapidly digested. A sample of the Hoffman-La Roche edestin, however, was not digested at the same rate as crystalline edestin prepared in the laboratory.

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[1] These washed corpuscles can be stored frozen and after being dialyzed and acidified can be used for the estimation of pepsin instead of the purified hemoglobin solution already described (Anson and Mirsky, 1932) which is more difficult to prepare. The one acid hemoglobin solution which we have prepared from frozen corpuscles was digested at the same rate as the purified hemoglobin.

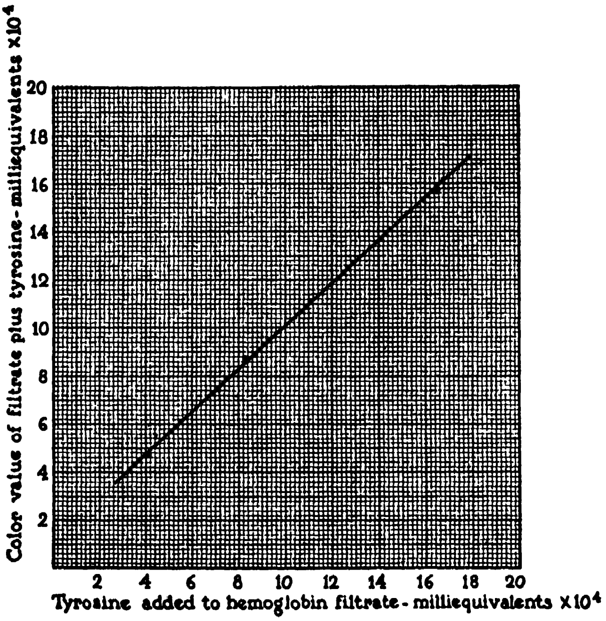

FIG. 1. Color values of various amounts of tyrosine dissolved in hemoglobin filtrates.

Calculations

What is measured is the color value of 5 ml. of the trichloracetic acid filtrate from digested hemoglobin in terms of the amount of tyrosine which would give the same color under the same conditions. For the purposes of calibration it must, therefore, first be determined how much color would be given by various known amounts of tyrosine in the trichloracetic acid filtrate from undigested hemoglobin which contains in addition to trichloracetic acid, phosphate and urea a small amount of color-producing substance not precipitated by trichloracetic acid.

10 parts 5 per cent trichloracetic acid are added to a mixture of 5 parts hemoglobin solution and 1 part water. To 5 ml. portions of the filtrate are added 1 ml. portions of 0.1 N hydrochloric acid containing various amounts of tyrosine. The colors developed with sodium hydroxide and the phenol reagent are read against the color developed from 0.15 mg. or 0.00083 milliequivalents, tyrosine dissolved in 5 ml. 0.2 N hydrochloric acid plus 1 ml. water. Fig. 1 shows how many

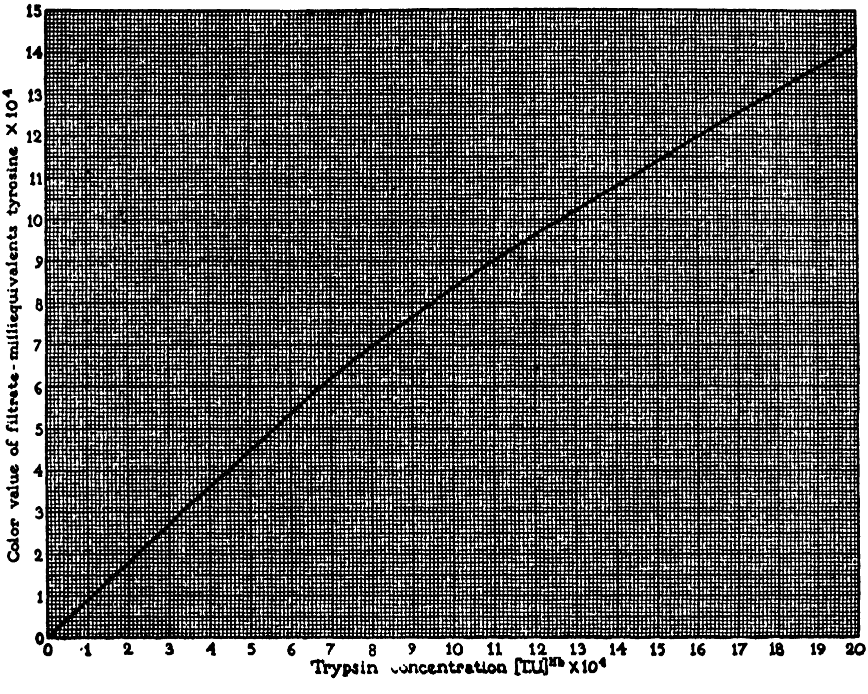

FIG. 2. Relation of trypsin concentration to color value of digestion products, 5 ml. filtrate, 5 minutes digestion at 25°C.

milliequivalents of tyrosine in hydrochloric acid are needed to give the same color as any given amount of tyrosine dissolved in the trichloracetic acid filtrate.

Digestion is now carried out with various amounts of enzyme. Fig. 2 gives the color values of the filtrates in terms of the amounts of tyrosine in the filtrate which would give the same colors. In practice since the properties of the hemoglobin solution are constant one avoids calculations by using a curve in which the amounts of trypsin are plotted directly against the colorimetric readings when the standard is set at 20. One does not have to use different calibration curves if different periods of digestion are used because increasing the digestion time *n* times is always equivalent to increasing the enzyme concentration *n* times.

For the purpose of using the hemoglobin-urea procedure the trypsin units may be considered as arbitrary numbers which are proportional to the amounts of trypsin which give the amounts of color-producing substances expressed by the curve. In order, however, to make the hemoglobin trypsin unit comparable with other units of proteolytic activity (Northrop, 1932; Anson and Mirsky, 1932) the following definition has been adopted. One unit of trypsin produces in 1 minute at 35.5°C. in 6 ml. of the digestion mixture an amount of color-producing substance not precipitable with trichloracetic acid which gives the same color as 1 milliequivalent of tyrosine. This definition assumes that the extent of digestion is proportional to the concentration of enzyme and to the time of digestion. These assumptions are correct only when the amount of digestion is small since as digestion proceeds the trypsin is inhibited by the products of digestion. The slope of the curve of Fig. 2 for small amounts of digestion is $1/5 \times 16/5 \times 1.75$ or 1.12 times less steep than it would be if the determination were carried out as described in the definition because the digestion is carried out for 5 minutes instead of 1, only 5 ml. of filtrate are used in the colorimetric estimation instead of the total 16 and the digestion is carried out at 25°C. instead of at 35.5°C. at which it is 1.75 times faster.

Effect of Variations in the Composition of the Digestion Mixture on the Extent of Digestion

Hemoglobins from the bloods of different individual animals are digested at the same rate. Doubling the hemoglobin concentration or reducing it 10 per cent has no detectable effect. The amount of urea can be increased or decreased 5 per cent, or the equivalent of 1 ml. of 0.1 N hydrochloric acid, 0.1 N sodium hydroxide or 10 per cent glycerol can be added to the digestion mixture without changing the extent of digestion 3 per cent.

Preparation of Purified Trypsin for the Standardization of Non-

Reproducible Proteins.—1 gm. of Fairchild's trypsin is suspended in 25 ml. 0.1 N hydrochloric acid, heated for 1 minute at 80°C. and cooled rapidly to room temperature with ice water. After 10 minutes, 6 gm. of ammonium sulfate are added and the suspension filtered. To each 10 ml. of the filtrate are added 2 gm. ammonium sulfate. The resulting precipitate is centrifuged and dissolved in enough 0.005 N hydrochloric acid to make the final volume 25 ml. This final solution has about 0.01 activity unit per ml.; *i.e.*, it has to be diluted about 10 times for estimation.

SUMMARY

The formation from hemoglobin of split products not precipitable by trichloracetic acid is taken as a measure of tryptic activity. The split products are estimated colorimetrically.

Many measurements of tryptic activity can be made in a short time and different samples of hemoglobin yield the same results.

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THE ESTIMATION OF ACTIVE NATIVE TRYPSIN IN THE PRESENCE OF INACTIVE DENATURED TRYPSIN

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Trypsin, which can catalyze the hydrolysis of proteins, is itself a protein (Northrop and Kunitz, 1932). The denaturation of proteins is reversible (Anson and Mirsky, 1931). When trypsin is denatured its proteolytic activity is completely lost. When the denaturation of trypsin is reversed the original proteolytic activity is completely restored (Northrop, 1932). The denaturation of trypsin and its reversal can therefore be followed by measurements of proteolytic activity. The present paper describes a technique for the measurement of the activity of native trypsin in the presence of inactive, denatured trypsin. Later papers will describe the application of this technique to the study of the effect of various denaturing agents such as heat, acid, alcohol and urea on the equilibria between native and denatured trypsin and on the rates of the denaturation of trypsin and its reversal.

To estimate trypsin the enzyme is added to a solution of protein and the rate of digestion is measured. In all the protein solutions which have hitherto been used reversal of the inactivation and denaturation of trypsin take place. If active, native trypsin is to be estimated in the presence of inactive, denatured trypsin, such change of inactive into active trypsin during the very estimation must obviously be avoided. This we have succeeded in doing by adding a suitable amount of the denaturing agent, urea, to the digestion mixture.

We have already described a method for the estimation of trypsin with hemoglobin (Anson and Mirsky, 1933) which can be used when the reversal of inactivation need not be considered. Digestion of denatured hemoglobin by trypsin is allowed to take place in a slightly alkaline solution buffered with phosphate. Precipitation of the denatured hemoglobin is prevented by urea. The undigested hemoglobin is precipitated with trichloracetic acid. The digested hemoglobin not precipitated by trichloracetic acid, which is a measure of the amount of trypsin used, is estimated by the blue color it gives with the phenol reagent. Considerable amounts of acid, alkali or glycerol can be added with the enzyme without any effect on the rate of digestion.

The change of inactive into active trypsin which takes place in the standard hemoglobin solution used for the ordinary estimation of trypsin can be prevented by adding more urea or (as is done in the procedure to be described) more alkali. If too much urea or too much alkali is added not only is the change from inactive to active trypsin prevented but the active trypsin is inactivated so fast that no measurement of activity is possible. In a digestion mixture in which there is neither reactivation nor a too rapid inactivation the rate of digestion is slower than it is in the standard hemoglobin solution used for the ordinary estimation of trypsin and the rate of digestion is much more sensitive to small changes in pH and urea concentration.

The general method of preventing reactivation during an analytical procedure by having present an inactivating agent such as urea may prove to be of use not only in the study of the denaturation of pure proteins but in testing for the protein nature of biologically active substances which have not been isolated but which are present in extremely dilute solutions together with many impurities. One would expect a protein substance regardless of its concentration or of the presence of impurities to lose its activity if exposed to denaturation procedures or to the proteolytic activity of trypsin. Trypsin, itself, however, which is a protein, can be heated in acid to 100°C. without any permanent loss of activity, for the denaturation and inactivation which take place on heating are reversed on cooling. There are proteins like hemoglobin which in their native form are not attacked by trypsin although in their denatured form they are rapidly digested. If a substance does not lose its activity when exposed to a denaturation procedure or to trypsin it may mean, therefore, not that the substance is not a protein but that there has been, as in the case of trypsin, reversal of denaturation or that, as in the case of hemoglobin, the protein must be denatured to be digested by trypsin. To make sure that there has been no inactivation by denaturation the activity should be measured under conditions which prevent the reversal of denaturation. To make sure that the substance is not digestible by trypsin the substance should first be exposed to conditions known to cause denaturation and then brought to the slightly alkaline solution suitable for tryptic digestion in the presence of some substance such as urea which will keep the substance denatured.

The Procedure.—5 ml. of the hemoglobin solution to be described are poured from a 175 × 20 mm. test-tube into 1 ml. of the enzyme solution in a second tube, the solution is poured back and forth, and the two test-tubes are placed in a water bath at 25° C. Sometime during the digestion period the small amount of solution in the test-tube from which the digestion mixture was last poured is drained into the other test-tube. After 5 minutes 10 ml. 5 per cent trichloracetic acid are added, the suspension is mixed with the few drops of digestion mixture still left in the third tube, allowed to stand 5 minutes and filtered. To 5 ml. of filtrate are added 10 ml. of 0.50 N sodium hydroxide and 3 ml. of the phenol reagent (Folin and Ciocalteau, 1927) diluted three times are added dropwise with stirring and always in the same way. After 1 to 10 minutes the blue color is read against the color developed from 0.00083 milliequivalents (0.15 mg.) of tyrosine dissolved in 5 ml. of 0.2 N hydrochloric acid containing 0.5 per cent formaldehyde. If the colorimetric comparison is made in the fairly monochromatic red light transmitted by the Corning glass filter No. 241 almost any blue glass or a blue solution can be used as a standard.

Preparation of the Hemoglobin Solution.—Defibrinated bovine blood is centrifuged, the serum and white corpuscles are siphoned off and the red corpuscles are washed once with an equal volume of 0.9 per cent sodium chloride solution. Water is added to give a solution containing in 100 ml. 10.5 gm. hemoglobin or 1.86 gm. nitrogen. This solution is stored frozen in paraffined paper ice-cream containers.

150 ml. of 10.5 per cent hemoglobin plus 7.5 ml. 1 N sodium hydroxide at 50–60°C. are added to 900 ml. of water at 100°C. There is then added with mechanical stirring 18 ml. of a solution 5 M in respect to sodium chloride and 0.5 M in respect to KH_2PO_4. The precipitate is filtered, washed and made up to 390 gm. with water. 390 gm. of urea and then 20 cc. 1 N sodium hydroxide are added and the solution is brought to room temperature to insure the complete solution of the hemoglobin. Finally, 160 ml. of 0.5 M boric acid and 430 ml. water are added and the solution is stored at 5°C. with toluol as a preservative. The

solution is the same as would be obtained by adding 39 gm. of urea to 100 gm. of a solution containing 1.5 gm. denatured hemoglobin and the equivalents of 80 ml. 0.1 M boric acid and 20 ml. 0.1 M sodium hydroxide.

Calibration.—Digestion is carried out with various dilutions of a trypsin solution whose activity in terms of trypsin units has been measured by the standard hemoglobin method. The colors developed from 5 ml. of trichloracetic acid filtrate are read against the color developed from 5 ml. of the standard tyrosine solution. Fig. 1 gives the relation between the trypsin units and the colorimetric readings when the standard is set at 20. The hemoglobin solution may be kept at least 2 months without any change in the calibration curve and different preparations of the hemoglobin solution yield the same calibration curve.

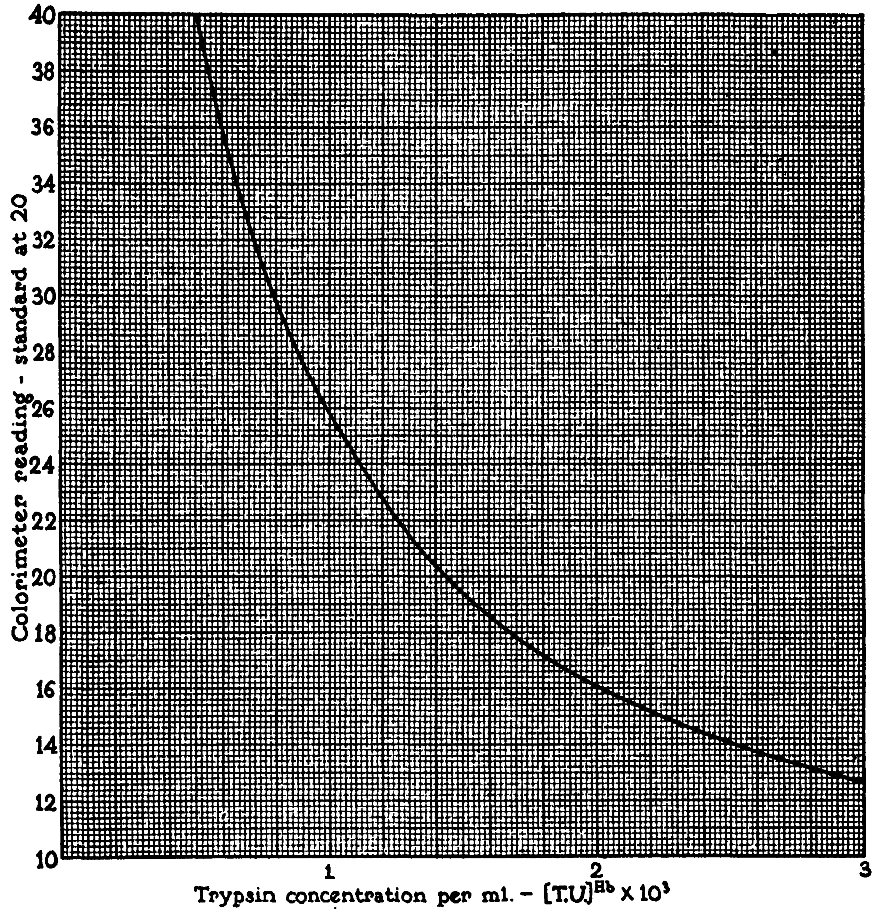

FIG. 1. Relation of trypsin concentration to color value of digestion products.

The Effect of Variations in the Composition of the Digestion Mixture on the Rate of Digestion.—Increasing the urea concentration 5 per cent reduces the extent of digestion 2 per cent. Decreasing the urea concentration 5 per cent increases the extent of digestion 2 per cent. The addition to the digestion mixture of the equivalent of 1 ml. of 5 per cent glycerol causes an increase of 6 per cent in the digestion; 1 ml. of 0.06 N hydrochloric acid causes an increase of 4 per cent; and 1 ml. of 0.06 N sodium hydroxide causes a decrease of 6 per cent. If more than 0.01 N acid or alkali is added with the enzyme then 0.5 ml. instead of 1 ml. of enzyme solution is used and there is added to the 5 ml. of hemoglobin solution 0.5 ml. of a solution which exactly neutralizes the acid or alkali added with the enzyme.

Evidence of the Prevention of the Reversal of Denaturation.—When trypsin is heated to 60°C. in 0.01 hydrochloric acid for 2 minutes it is completely inactivated and denatured. On cooling the solution the original activity is restored. If 5 ml. of the hemoglobin solution plus 0.5 ml. of water are poured into 0.5 ml. of a hot 0.01 N hydrochloric acid solution of trypsin which before heating contained 24×10^{-3} units of active enzyme and digestion is carried out for 10 minutes only 0.65×10^{-3} units of activity are found.

When trypsin is heated or cooled to 45°C. in 0.01 N hydrochloric acid it is about half inactivated and denatured. The activity of such a mixture is the same whether or not the denatured half of the trypsin is first precipitated with salt and removed. The experiment is carried out as follows: Into 0.5 ml. of trypsin (1.72×10^{-2} $[\text{T.U.}]^{\text{Hb}}$) in 0.01 hydrochloric acid heated to 45°C. for 5 minutes are poured 5 ml. hemoglobin solution plus 0.5 ml. water. 1 ml. of the mixture is immediately added to 5 ml. of a mixture of 5 parts hemoglobin solution and 1 part water. The digestion is carried out for 10 minutes after the hemoglobin solution was first poured into the enzyme solution. For the estimation with salt, 2 ml. of 5 M sodium chloride in 0.01 N hydrochloric acid are poured into 2 ml. of the heated trypsin solution. The resulting suspension is centrifuged. 1 ml. of the supernatant solution is added to 5 ml. hemoglobin solution; 1 ml. of this mixture is then immediately added to 5 ml. of a mixture of 5 parts hemoglobin solution to 1 part water and the digestion is carried out as before. By both methods the heated solution is found to have 0.86×10^{-2} units of activity.

Effect of Variations in the Composition of the Digestion Mixture on the Extent of the Reversal of Inactivation.—The less urea in the digestion mixture the more reversal of inactivation. If the amount of urea is decreased 5 per cent the amount of reversal is still less than 5 per cent. If the amount of urea is decreased a third about 20 per cent reversal takes place. The results under these conditions are not very reproducible. Acid favors reversal, alkali the reverse, but the addition of 1 ml. of 0.04 N hydrochloric acid or sodium hydroxide (which is more than permissible if the rate of digestion by active trypsin is to be kept constant) has no significant effect on the extent of reversal. Glycerol favors reversal. The addition of the equivalent of 1 ml. of 5 per cent glycerol to the digestion mixture increases the amount of reversal 60 per cent.

SUMMARY

Inactive denatured trypsin changes into active native trypsin in the protein solutions which have been used to estimate tryptic activity. If the digestion mixture, however, is alkaline enough and contains enough urea this change does not take place. Such a digestion mixture can be used to estimate active native trypsin in the presence of inactive denatured trypsin.

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ABSORPTION OF PEPSIN BY CRYSTALLINE PROTEINS

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Pepsin, like other enzymes, is removed more or less completely from solution by various insoluble substances. Dauwe (1) showed that insoluble proteins were particularly efficient in this respect. These results were confirmed by Abderhalden and coworkers (2). The writer found (3) that the quantity of pepsin removed by insoluble proteins depended largely upon the pH of the solution and that under certain conditions the ratio of the enzyme in the precipitate to that in the solution was the same as the chloride ion ratio. This result suggested that the pepsin was a negative ion and was distributed like any other ion in accordance with the Donnan equilibrium (4). In the acid range, however, between pH 2.0 and 5.0 the results were anomalous from this point of view since much more pepsin was absorbed than would be expected from the Donnan equilibrium.

It has recently been found by Dyckerhoff and Tewes (5) and by Waldschmidt-Leitz and Kofrányi (6) that crystalline proteins such as edestin or melon globulin also possess the property of absorbing pepsin from pepsin solutions, and Waldschmidt-Leitz considers that the crystalline foreign protein removes the active group from the pepsin protein. If this explanation were correct a convenient means would be at hand to separate the active group of pepsin from the protein-pepsin molecule, since the foreign protein (edestin or melon globulin) is rapidly and completely digested by pepsin and since there is little or no loss in activity during peptic digestion. It would only be necessary, therefore, to allow the complex of foreign protein and pepsin to digest until all the protein had been destroyed and the active pepsin must then be found in solution free from protein. When this experiment is performed, however, it is found that there is left in the digested edestin solution an amount of pepsin protein just equivalent to the peptic activity present and equivalent to the loss in pepsin protein from the original pepsin solution. The absorption of pepsin by crystalline foreign protein, therefore, consists in the absorption of the pepsin protein, as such, and does not separate the pepsin protein into an inert protein and an active pepsin group.

The absorption of pepsin by edestin shows a sharp maximum at about pH 4.0 and pepsin may be removed completely from dilute solutions by stirring with a suspension of edestin crystals at this pH. The pepsin protein may be recovered from the "edestin-pepsin" complex by allowing the "edestin-pepsin" to autolyze, or by simply extracting the edestin-pepsin with N/4 sulfuric acid at 0°C. The recovered pepsin may be identified by its tyrosine-tryptophane content which is twice that of edestin and by its content of basic nitrogen which is about one-quarter that of edestin. It may be readily recrystallized and obtained in the characteristic crystalline form and with the characteristic specific activity of the original crystalline pepsin. If a suspension of edestin crystals at pH 4.0 is added to increasingly concentrated solutions of either crystalline or crude pepsin surprisingly large amounts of pepsin are taken up by the edestin crystals and preparations may be obtained which contain nearly 50 per cent pepsin and are, therefore, one-half as active as crystalline pepsin itself and much more active than commercial pepsin preparations. The general form and appearance of the edestin crystals is not markedly changed, but if the suspension of edestin crystals in the pepsin solution is allowed to stand for several hours at room temperature the edestin gradually dissolves and the pepsin content of the remaining precipitate increases. On longer standing the precipitate becomes less and less in bulk and finally dissolves completely, so that the final result is a solution of digested edestin containing the original quantity of pepsin.

The rate of autolysis can be increased by dissolving the edestin-pepsin precipitate in hydrochloric acid. The edestin protein is then very rapidly destroyed and there is left the pepsin protein. There is no change in activity during this process so that the autolysis of "edestin-pepsin" differs strikingly from the autolysis of pepsin itself, since in the latter case the destruction of the protein is paralleled by a corresponding loss in activity while in the case of "edestin-pepsin" the edestin is destroyed without any corresponding loss in activity. There is, therefore, no reason to consider the "edestin-pepsin" complex as having any activity of its own aside from that due to the content of pepsin protein.

If supersaturated solutions of autolyzed edestin-pepsin or autolyzed solutions of pepsin alone are allowed to stand, the pepsin precipitates out in the form of spheroids, as Dyckerhoff and Tewes have shown (5). It is characteristic of proteins to appear in this spheroidal form when conditions are not quite right for crystallization or when they have not been sufficiently purified. The pepsin spheroids consist largely of pepsin but contain from 10 to 30 per cent non-protein nitrogen. They may be purified by solution and precipitation with acid or magnesium sulfate and the pepsin may then be obtained in the usual crystalline form.

The edestin-pepsin complex may also be formed by mixing cold solutions of edestin with solutions of pepsin. A precipitate forms which varies in composition and quantity with the pH of the solution. The maximum quantity and the maximum activity again are found at about pH 4.0. If the relative concentrations of pepsin and edestin are varied at pH 4.0 the quantity of pepsin in the precipitate is a maximum when equal concentrations (by weight) of pepsin and edestin are mixed. Under these conditions the precipitate contains nearly 75 per cent pepsin and is about three-quarters as active as crystalline pepsin itself.

Since pepsin and edestin both have an equivalent weight of about 1,000 the precipitate having maximum activity corresponds approximately to three equivalents of pepsin to one of edestin. Since the molecular weight of pepsin is only one-sixth that of edestin this corresponds approximately to eighteen molecules of pepsin to one of edestin.

Similar experiments may be performed with the globulin from melon seed (*Cucumis*), as Waldschmidt-Leitz and Kofrányi (6) have found, and also with gelatin. In both cases the pepsin protein removed from the pepsin solution and taken up by the solid protein corresponds to the loss of activity of the solution.

EXPERIMENTAL RESULTS

I. Absorption of Pepsin by Edestin Crystals from Crystalline Pepsin Solutions at Various pH

Experimental Procedure.—0.1 per cent solution of crystalline pepsin was titrated to various pH with sulfuric acid, cooled to 0°C. and 1 gm. crystalline edestin[1] (La Roche) was then added to 25 ml. of the solutions. The suspensions were stirred for a few minutes and kept at 6°C. for 18 hours. The suspensions were then centrifuged, the precipitates washed with 10 ml. of water and dissolved in 25 ml. of N/20 hydrochloric acid. Samples of the original suspensions, the supernatant solutions and the solutions of the precipitates were then analyzed for pepsin nitrogen, total nitrogen, tyrosine equivalent and peptic activity by the hemoglobin method (7). Pepsin nitrogen was determined by titrating the samples to pH 2.0 with hydrochloric acid and keeping at 33°C. for 3 hours. Any foreign protein is digested under these conditions and the protein nitrogen remaining is determined as usual by precipitation with hot trichloracetic acid. This protein nitrogen is called pepsin nitrogen. It is essential that hot trichloracetic acid be added to the cold pepsin solution as otherwise the pepsin may autolyze while the solution is being heated.

The tyrosine equivalent is determined by the development of the blue color with Folin's reagent; 3 ml. of the solution is treated with the reagent and the measurement carried out exactly as for the hemoglobin filtrate in the pepsin determination with hemoglobin. This gives the number of milligrams of tyrosine which would give the same color as the tyrosine plus tryptophane contained in the unknown solution.

The results of the analyses have been calculated to the basis of 1 ml. of the original suspension.

The results of the experiment are shown in Table I and Fig. 1. There is a sharp maximum of absorption at about pH 4.0 and in this range 90 per cent of the activity is found in the precipitate. Correspondingly about 90 per cent of the pepsin nitrogen is also in the precipitate. The pepsin nitrogen is identified by the fact that it is not digested if allowed to stand in acid solution and by its content of tyrosine plus tryptophane (tyrosine equivalent) of about 0.64 mg. tyrosine per mg. pepsin nitrogen, while the tyrosine equivalent of edestin is about 0.34. In this experiment, owing to the low concentration of pepsin the specific activity of the precipitate is low and is only about 3 or 4 per cent that of the crystalline pepsin.

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[1] 1 gm. of this preparation contained 0.65 gm. dry edestin, as calculated from the nitrogen content.

TABLE I

Absorption of Pepsin by Edestin from Crystalline Pepsin Solutions at Various pH

pH		1.12	1.6	2.8	3.63	4.25	4.85	5.5	5.6	5.9	Control no edestin 4.5	Control no pepsin 5.9
Concentration H_2SO_4 — N		0.2	0.10	0.05	0.025	0.012	0.006	0.003	0.0015	0	0	0
$[P.\ U.]^{Hb}_{ml.}$	Suspension	0.028	0.027	0.027	0.026	0.028	0.028	0.027	0.026	0.027	0.028	0
	Supernatant	0.025	0.025	0.011	0.0006	0.0026	0.010	0.018	0.020	0.021	0.028	0
	Precipitate	0.0016	0.002	0.016	0.025	0.025	0.014	0.008	0.006	0.005	0	0
Per cent activity in precipitate		6	7.4	59	93	93	52	29	22	18	0	
Pepsin N/ml., *mg.*	Supernatant	0.14		0.056	<0.01	0.017	0.056	0.084	0.110		0.17	
	Precipitate	0.02		0.10	0.15	0.14	0.09	0.06	0.04			
Per cent total pepsin N in precipitate		12		63	94	87	56	37	25			
$[P.\ U.]^{Hb}_{mg.\ pepsin\ N}$	Supernatant	0.18		0.20		0.15	0.18	0.21	0.18		0.17	
	Precipitate	0.08		0.16	0.17	0.18	0.16	0.13	0.15			
Tyrosine equivalent per mg. pepsin N	Supernatant	0.56		0.54			0.54	0.64	0.64		0.60	0.34
	Precipitate				0.58	0.60						
Total N/ml., *mg.*	Supernatant	4.3		3.1	1.36	0.80	0.52	0.41	0.40	0.38	0.20	
	Precipitate (by difference)	0.7		1.9	3.6	4.2	4.5	4.6	4.6	4.6		4.8
$[P.\ U.]_{mg.\ N}$	Supernatant	0.006		0.004	0.0004	0.003	0.02	0.045	0.05	0.05	0.14	0
	Precipitate	0.002		0.0085	0.007	0.006	0.003	0.0017	0.0012	0.001		

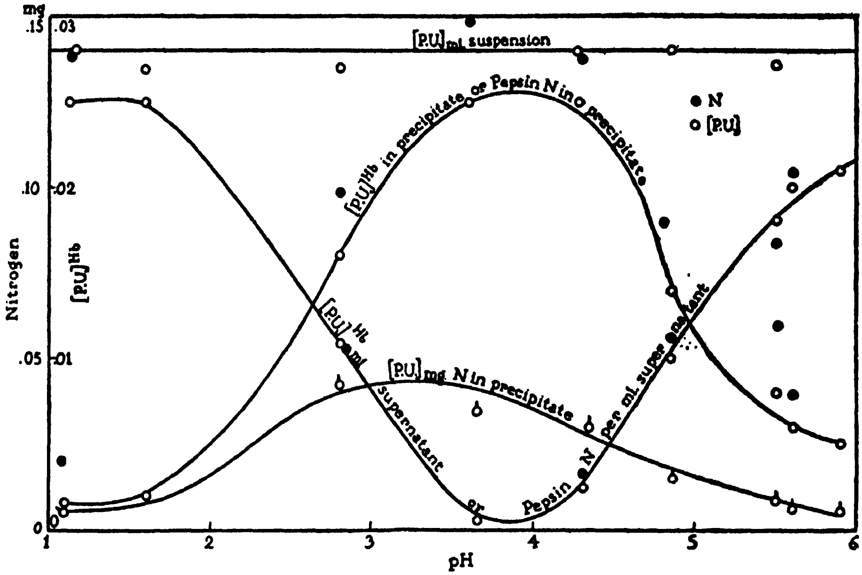

FIG. 1. Absorption of pepsin by crystalline edestin at various pH

II. Recovery of Crystalline Pepsin from Edestin-Pepsin Complex

Experimental Procedure.—

	Fraction No.
1000 gm. of Parke, Davis pepsin dissolved in 2500 ml. of water, pH about 5.0....	1
The solution cooled to 6°C. and 40 gm. crystalline edestin (La Roche) added, the suspension stirred for about 2 hours, filtered through fluted paper and the precipitate washed twice with water. Precipitate consisted of slightly deformed edestin crystals. The precipitate was suspended in water and titrated with hydrochloric acid to pH 2.5 and kept at 20°C. for 24 hours.....	2
A slight flocculent precipitate formed which was filtered off and suspended in N/50 hydrochloric acid..	3
Filtrate allowed to stand at 6°C. for 3 weeks. Dark, oily gum settled on the bottom; insoluble in alkali or acid and contained very little activity. Supernatant solution decanted from this gum and the protein precipitated by the addition of 1 volume of saturated magnesium sulfate and filtered. The precipitate was suspended in 8 ml. of water, warmed to 45°C. and dissolved by the addition of a few drops of N/10 sodium acetate...........	7
N/2 sulfuric acid added until slight cloud appeared and the solution allowed to cool slowly. Precipitate consisted of spheroids mixed with some amorphous material and a few pepsin crystals..........................	8

Precipitate redissolved at 45°C. and recrystallized as above. Precipitate consists of pepsin crystals; dissolved in pH 5.0 sodium acetate	11
Filtrate	10
This filtrate was allowed to stand at 6°C. and after 2 weeks most of the pepsin had crystallized out	

The various fractions obtained in this way were analyzed for total nitrogen, pepsin nitrogen, tyrosine equivalent, and basic nitrogen and their activities determined by a series of methods (2). Basic nitrogen is determined by dissolving the sample in 5 M hydrochloric acid and heating in the autoclave at 120°C. for 2 hours. Total nitrogen is then determined on 1 ml. of this solution; 2 ml. of solution is added to 2 ml. of a saturated solution of phosphotungstic acid in 5 M hydrochloric acid and the mixture left 18 hours at 0°C. It is centrifuged and the total nitrogen determined on 2 ml. of the supernatant solution. The difference between this figure and the total nitrogen is the basic nitrogen.

The results are tabulated in Table II and are expressed as the total nitrogen or activity present in the entire fraction. The results show that in this case about 1 per cent of the total activity and also of the pepsin nitrogen was taken up by the edestin. The edestin-pepsin had about one-half the activity of the original Parke, Davis pepsin on the basis of total nitrogen content, as determined by any of the methods used, except the gelatin viscosity method. The activity by the gelatin viscosity method is the same as that of the Parke, Davis pepsin so that the edestin has a slightly preferential affinity for the gelatinase fraction. After autolysis and precipitation with magnesium sulfate most of the activity is found in the precipitate which now has about one-half the specific activity, on a total nitrogen basis, of the crystalline pepsin. This precipitate of amorphous pepsin when dissolved with alkali and then acidified and allowed to cool appears in the form of spheroids ("*kugeln*") mixed with a few crystals which have about the same specific activity as the amorphous precipitate. When these spheroids are dissolved and crystallized in the usual way normal pepsin crystals are obtained with the same specific activity as the ordinary crystalline pepsin except that they contain considerable gelatinase, as shown by higher specific activity as measured by the viscosity of gelatin. Once crystallized pepsin always contains more or less of this gelatinase fraction and four or five crystallizations are required to free the pepsin completely from the gelatin-splitting enzyme (9).

TABLE II

Analysis of Various Fractions

	Method	P. D. pepsin	"Edestin-pepsin"	Amorphous pepsin	Spheroids	Filtrate	Pepsin crystals	5 × crystalline pepsin	Edestin
Fraction No.		1	2	7	8	10	11	5/9/32	
Total N, *mg.*		200,000	5000	116	35	10	11		
Total pepsin N, *mg.*		20,000	210	84	28	5	10		
$[P. U.]^{Hb}$ total		3000	35	12	4.2	0.7	1.6		
$[P. U.]_{mg. N}$	Hb	0.015	0.007	0.10	0.12	0.07	0.15	0.20	
	Gel. V^-	2	2	12	12.7	6.2	28.5	11.0	
	Cas. V^-	150	66	1000	1100	700	1450	1700	
	Ed. V^-	170	72	1000	950	786	1250	1650	
	Cas. S.	0.05	0.02	0.36	0.33	0.27	0.5	0.5	
	Cas. V^+	30	13	180	160	160	200	310	
	Ren.	4×10^4	1.6×10^4	50×10^4	30×10^4	20×10^4	60×10^4	44×10^4	
$[P. U.]_{mg. pepsin N}$	Hb	0.15	0.16	0.15	0.15	0.14	0.16	0.20	
	Gel. V^-	18	40	16	17	12	31	11	
	Cas. V^-	1400	1500	1450	1500	1500	1500	1700	
	Ed. V^-	1500	1500	1320	1300	1400	1360	1650	
	Cas. S.	0.47	0.5	0.5	0.46	0.54	0.54	0.5	
	Cas. V^+	280	290	250	230	300	220	310	
	Ren.	36×10^4	38×10^4	60×10^4	40×10^4	40×10^4	50×10^4	44×10^4	
Tyrosine equivalent per mg. pepsin N		0.5		0.48	0.50	0.56	0.56	0.67	0.32
Basic N as per cent pepsin N		8	7.5				6.7	8	35

When the specific activity is calculated on the basis of the pepsin nitrogen content, *i.e.* protein nitrogen which is not destroyed upon standing in acid solution, the specific activity remains constant throughout all fractionations, as shown by the lower part of the table.[2] In other words, the content of pepsin protein present in every fraction is just sufficient to account for the observed activity. The fact that this protein nitrogen is really pepsin nitrogen and not edestin nor one of its decomposition products is shown by the fact that the tyrosine equivalent and the basic nitrogen content is that of pepsin and not of edestin. Identification is made complete by the actual recovery of typical pepsin crystals. The per cent actually recovered in crystalline form is rather small but is about what would be expected from a solution containing such a large amount of protein decomposition products.

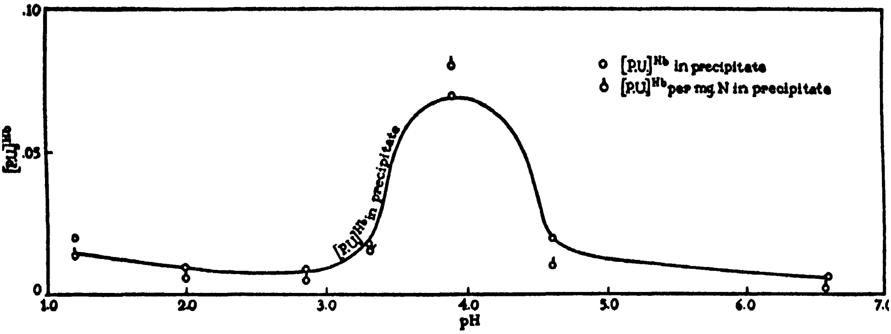

FIG. 2. Absorption of pepsin by crystalline edestin from solutions of Cudahy pepsin at various pH.

The method of determining activity by formol titration of Dyckerhoff and Tewes was not used in general since it is very troublesome and inaccurate but the results with this method agree approximately with those given by Dyckerhoff and Tewes, as shown in Table III. The casein solution used was made up as described by Dyckerhoff and Tewes (5) for their "pH 4.0 casein." However, the pH of this solution, as determined by the hydrogen electrode or quinhydrone electrode is about 2.35, although methyl-orange gives an apparent pH of about 4.0. Dyckerhoff and Tewes used the indicator method[3] for determining pH and their pH values are from 1 to 1.5 pH more alkaline than would be found by the hydrogen electrode method. This probably accounts also for the very alkaline optimum pH of digestion reported by these workers. Under the conditions used by Dyckerhoff and Tewes the casein is digested practically instantly, owing to the enormous concentration of pepsin added and the titration obtained represents the final stages of the digestion. Under these conditions the change in titration with increasing pepsin concentration is very slight so that there is a very large error. On the other hand, if the first part of the curve is used the increase in titration is extremely small and difficult to determine. The large quantity of enzyme used renders it difficult to obtain the initial titration since the reaction proceeds extremely rapidly at first and also renders it difficult to be sure that there is no change in pH of the casein solution caused by the addition of the pepsin. A number of very erratic results were obtained at first and were traced to marked changes in pH in the casein solution upon the addition of the pepsin. The table shows, however, that the activity of the various preparations agrees quite closely with that reported by Dyckerhoff and Tewes.

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[2] It may be noted that the specific activity of this sample of Parke, Davis pepsin was only about one-half that usually found and also that the tyrosine equivalent per mg. nitrogen is slightly low. Both these results are due to the presence of an inert protein in this particular sample which is evidently carried through the adsorption procedure. This inert protein may be removed by allowing the solutions to stand longer in acid solution, but since there is a slight loss of activity under this condition this procedure could not be used for analytical purposes. The activity by some methods, especially edestin viscosity, differs from that previously obtained (8) owing to variations in the edestin solution.

It will be noted that 24 mg. of enzyme gives only about 0.2 ml. more titration in 24 hours than 6 mg. of enzyme, *i.e.* a difference of 400 per cent in the pepsin concentration makes a difference in titration of only 0.2 ml. so that since the error in the titration is about ± 0.05 ml. there is an uncertainty of nearly 100 per cent in the method. The preparation of Cudahy pepsin used by Dyckerhoff and Tewes was apparently slightly more active than that used in the present experiments.

III. Extraction of Pepsin from "Edestin-Pepsin" with Sulfuric Acid

If edestin-pepsin prepared by absorbing pepsin with crystalline edestin is stirred at 0°C. with sulfuric acid at pH about 1.0, the pepsin dissolves out leaving inactive edestin crystals. The results of such an experiment are shown in Table IV. A preparation of "edestin-pepsin" of relatively low activity was used purposely since it would be expected that a small amount of pepsin would be removed with more difficulty than a larger amount. The experiment shows that 80 per cent of the activity and of the pepsin nitrogen is removed from the

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[3] Personal communication from Drs. Dyckerhoff and Tewes.

TABLE III

Hydrolysis of Casein by Various Pepsin Preparations

8 ml. casein ("pH 4.0" Dykerhoff and Tewes) + 2 ml. pepsin solution at 35°C. Formol titration on 2 ml.

Enzyme preparation	Crystalline pepsin		Cudahy U.S.P. 1:10,000		"Edestin-pepsin"		
Mg. enzyme/2 ml. enzyme solution....	2.0	2.0	9.0	9.0	24	6	6
$[\text{P. U.}]^{\text{Hb}}_{\text{ml. enzyme solution}}$............	0.030		0.030		0.030		
	J.H.N.	D. and T., Table 3	J.H.N.	D. and T., Table 4	J.H.N.	J.H.N.	D. and T., Table 12a, Table 1a
Δ time	Δ ml. N/20 sodium hydroxide per 2 ml.						
hrs.							
1	0.26		0.22	0.29	0.26	0.16	
3				0.39			0.18
4	0.36		0.33		0.34	0.23	
6		0.35		0.43			
24	0.52	0.50	0.52	0.73	0.56	0.32	0.35

"edestin-pepsin" by the first extraction with sulfuric acid. The three following extractions remove practically all the remaining activity and pepsin nitrogen so that finally a little more than 90 per cent of the total original activity is recovered in the washings. The concentration of pepsin nitrogen in the second, third and fourth washings and in the final solid are too small to be accurately determined owing to the difficulty of completely digesting such a large excess of inert protein. Attempts to separate the complex by extraction or washing at pH 5.0 to 6.0, where pepsin itself is very soluble and edestin is insoluble, were not successful. If anything, the specific activity of the precipitate increases. Evidently the pepsin edestin complex is less soluble than edestin alone.

TABLE IV

Extraction of Pepsin from Edestin-Pepsin

	Total N/ml.	Pepsin N/ml.	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.N}}$	$[\text{P. U.}]^{\text{Hb}}_{\text{mg. pepsin N}}$	pH
	mg.	*mg.*				
0.5 gm. "edestin-pepsin" + 10 ml. N/4 sulfuric acid.	10	0.30	0.05	0.005	0.17	0.8
Stir at 0°C. for 20 min.						
Centrifuge—supernatant	1.3	0.23	0.038	0.029	0.165	
Precipitate stirred + 10 ml. N/10 sulfuric acid.						
Centrifuge—supernatant	1.3	0.02	0.003	0.0025	0.15	
Precipitate stirred + 10 ml. N/10 sulfuric acid.						
Centrifuge—supernatant	1.4	0.02	0.0024	0.0017	0.12	
Precipitate stirred + 10 ml. N/10 sulfuric acid.						
Centrifuge—supernatant	1.2	0.02	0.0020	0.0016	0.10	
Precipitate + 10 ml. water—suspension edestin crystals	4.5	0.02	0.001	0.0002	0.05	
Total $[\text{P. U.}]^{\text{Hb}}$ in washings			0.454			
in precipitate			0.010			
			0.464			
Total original $[\text{P. U.}]^{\text{Hb}}$			0.50			

IV. Effect of the Concentration of Pepsin on the Absorption of Pepsin by Edestin at pH 4.0, 6°C. from Crystalline Pepsin Solutions

Experimental Procedure.—About 10 gm. of twice crystallized pepsin was stirred in 75 ml. of water and N/2 sodium hydroxide added until the solution was at pH 4.0 (clear solution). The solution was diluted with water to the concentrations noted in Table V, cooled to 6°C. and 20 ml. of the various dilutions added to a series of suspensions of 1 gm. of edestin in 5 ml. N/10 sulfuric acid in 250 ml. Erlenmeyer flasks; the suspensions were stirred occasionally and left at 6°C. for 18 hours. They were then centrifuged and the precipitates washed with 20 ml. cold water, dissolved in 25 ml. N/20 hydrochloric acid and allowed to stand at 24°C. for 24 hours. The solutions were then analyzed for peptic activity by the hemoglobin method, protein nitrogen, total nitrogen and tyrosine equivalent.

The results of the experiment are shown in Table V and Fig. 3. As the concentration of pepsin is increased the quantity of pepsin in the edestin crystals increases until a maximum value is reached which in this case corresponds to about 10 per cent pepsin. As usual, the pepsin protein taken up by the precipitate is just equivalent to the activity found in the precipitate. The pepsin protein has the characteristic tyrosine equivalent of pepsin and also the characteristic specific activity.[4]

V. Effect of the Pepsin Concentration on the Absorption of Pepsin by Crystalline Edestin from Solutions of Cudahy Pepsin at pH 4.0 at 6°C.

Experimental Procedure.—100 gm. Cudahy pepsin dissolved in 100 ml. water and the solution diluted with water to the concentrations noted and cooled to 6°C. 1 gm. of edestin in 5 ml. of N/10 sulfuric acid added to 20 ml. of the various pepsin solutions, stirred for 20 minutes and kept at 6°C. for 18 hours; pH of all solutions about 4.0. The supernatant was then centrifuged and the precipitate washed with 20 ml. water and dissolved in 25 ml. N/20 hydrochloric acid. The original suspension and the solution and precipitate were analyzed for total nitrogen, pepsin nitrogen and activity by the hemoglobin method.

The results have been calculated to the basis of 1 ml. of the original suspension and are given in Table VI and Fig. 3.

When Cudahy pepsin is used the activity of the precipitate increases to a maximum which, however, is considerably higher than was the case in the experiment with crystalline pepsin solutions. The pepsin

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[4] See footnote, page 173.

TABLE V

Effect of the Concentration of Pepsin on the Absorption of Pepsin by Edestin at pH 4.0, 6°C. from Crystalline Pepsin Solutions

Suspension	Total N/ml, *mg*	20.00	12.5	9.00	7.00	6.00	5.50	5.00	5.10	No pepsin	No edestin
	Pepsin N/ml., *mg*	15.00	7.5	3.75	1.87	0.93	0.47	0.24	0.12		
	$[P.U.]^{Hb}_{ml.}$	2.60	1.3	0.65	0.33	0.17	0.085	0.048	0.020		
Precipitate	$[P.U.]_{ml.}$	0.065	0.071	0.070	0.080	0.078	0.049	0.024	0.012		
	Per cent total activity in precipitate	2.5	5.5	11.00	24.0	46.0	57.0	50.0	60.0		
	Pepsin N/ml., *mg*	0.45	0.43	0.49	0.56	0.48	0.30	0.18	0.13		
	Total N/ml., *mg*	4.7	5.1	5.2	5.0	5.2	5.1	5.0	5.1	4.8	
	$P.U.]^{Hb}_{mg. N}$	0.014	0.014	0.014	0.016	0.015	0.01	0.005	0.0025		0.15
	$[P.U.]^{Hb}_{mg. pepsin N}$	0.145	0.17	0.14	0.14	0.16	0.16	0.14	0.10		0.16
	Tyrosine equivalent/mg. pepsin N	0.60	0.60	0.60	0.57	0.58	0.60	0.60		0.34	0.60
	Per cent pepsin in precipitate	10.0	9.0	10.0	11.0	9.0	6.0	3.6	2.6		
	Mols pepsin/mols edestin	0.66	0.59	0.66	0.74	0.52	0.38	0.22	0.16		

nitrogen absorbed is again equivalent to the activity taken up by the precipitate. It will be noted that in this experiment the specific activity of the precipitate, calculated to the basis of pepsin nitrogen, is about 0.2 which is the usual value for crystalline pepsin, whereas in the preceding experiment it was only about 0.16. This is due to the fact that the crystalline pepsin used in the previous experiment was prepared from a commercial preparation which was peculiar in that it contained about 20 per cent of an inert protein very similar to pepsin itself and which could only be removed by five or more fractional crystallizations or by prolonged standing in acid solution. The preparation used in the experiment referred to had not been purified by fractional crystallization since it seemed possible that absorption by edestin might serve as a means of removing this inert protein. The results, however, show that this is not the case but that the inert protein is absorbed by the edestin to the same extent as the active enzyme so that the specific activity is not changed by the edestin treatment. The specific activity on a total nitrogen basis of the

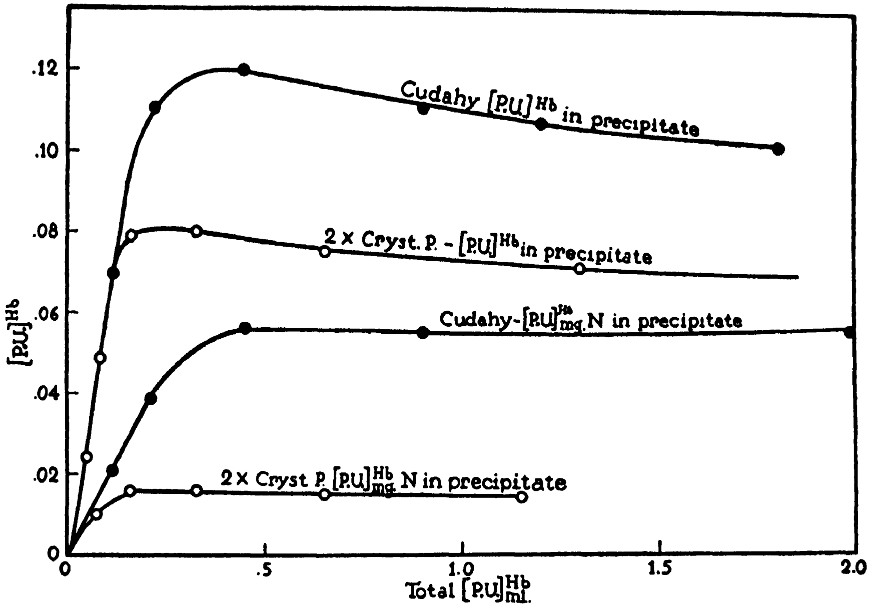

FIG. 3. Effect of the concentration of pepsin on the absorption of pepsin by edestin from solutions of crystalline pepsin or Cudahy pepsin.

TABLE VI

Effect of the Pepsin Concentration on the Absorption of Pepsin by Crystalline Edestin from Solution of Cudahy Pepsin at pH 4.0 at 6°C.

								No pepsin	No edestin
Suspension	Total N/ml., *mg.*	95	50	27	16	10.6	7.8	4.8	
	$[P.U.]^{Hb}_{ml.}$	3.6	1.8	0.90	0.45	0.22	0.11		
	Pepsin N/ml., *mg.*	18	9	4.5	2.2	1.1	0.55		
Precipitate	Protein N/ml., *mg.*	3.2	2	2	2.3	2.9	3.5	4.8	
	Pepsin N/ml., *mg.*	0.26	0.46	0.50	0.50	0.54	0.31		
	$[P.U.]^{Hb}_{ml.}$	0.056	0.10	0.11	0.12	0.11	0.068	0	
	Per cent total $[P.U.]^{Hb}$	1.5	5.5	12	27	50	62		
	$[P.U.]_{mg.\ protein\ N}$	0.017	0.05	0.055	0.052	0.038	0.02	0	0.04
	Per cent pepsin $\left(\frac{\text{Pepsin N}}{\text{Protein N}}\right)$	8.1	23	25	22	19	8.8		
	Mols pepsin/mols edestin	0.52	1.8	2.0	1.7	1.5	0.56		
	$[P.U.]^{Hb}_{mg.\ pepsin\ N}$	0.21	0.22	0.22	0.24	0.20	0.22	0	0.20

"edestin-pepsin" prepared with Cudahy solution is about one-third that of crystalline pepsin and is, therefore, nearly twice as high as that of the original Cudahy pepsin. This is near the maximum activity obtained in any experiment in which crystalline edestin was treated with pepsin solutions. It corresponds to about 30 per cent pepsin which is equivalent to about 3 moles of pepsin to 1 mole of edestin or about one-half an equivalent of pepsin per equivalent of edestin. The results of both experiments are shown in Fig. 3.

The low activity of the precipitate in the experiment with crystalline pepsin is due to some accidental condition, such as stirring or length of time in which the edestin was in contact with the sulfuric acid since in other experiments "edestin-pepsin" which had a much higher activity was prepared from crystalline pepsin solutions.

VI. Changes in Edestin-Pepsin Suspensions with Time

In the preceding experiments the edestin crystals were stirred with the pepsin solutions for a few minutes and then allowed to stand at 6°C. for 18 hours. In the present experiment the suspension was stirred for about 1 hour at 0°C. and then continued at 30°C. in order to accelerate the reaction so that it would be completed in a convenient time. The results of the experiment are shown in Fig. 4.

Experimental Procedure.—Pepsin solution, twice crystallized, pH 4.0. Edestin solution, crystalline (La Roche). 5 gm. edestin stirred with 20 ml. cold N/10 sulfuric acid and 80 ml. cold pepsin solution added. Suspension stirred at 0°C. for 1 hour and then at 35°C. 5 ml. samples centrifuged at intervals and precipitate dissolved in 5 ml. N/50 hydrochloric acid. Suspension, supernatant and precipitate analyzed for total nitrogen, total protein nitrogen, pepsin nitrogen and activity by hemoglobin method.

Total protein nitrogen: 1 ml. of solution plus 9 ml. boiling 5 per cent trichloracetic acid, cool, centrifuge, and wash precipitate with 5 per cent trichloracetic acid and nitrogen determined.

Pepsin nitrogen: 1 ml. plus 9 ml. N/20 hydrochloric acid, 37°C. for 3 hours. 5 ml. plus 5 ml. boiling 10 per cent trichloracetic acid and precipitate washed and analyzed for nitrogen.

Total protein nitrogen − pepsin nitrogen = edestin nitrogen.

Activity: Solution diluted to contain about 0.01 mg. pepsin N/ml. and activity determined by hemoglobin method.

All results calculated to the basis of 1 ml. of original suspension. Composition of original suspension calculated from analysis of pepsin and edestin alone.

	Total N	Pepsin N	Edestin N	$[\text{P. U.}]^{\text{Hb}}$
	mg.	*mg.*	*mg.*	
Pepsin	4.4	3.5		0.64
Edestin	7.6		7.6	

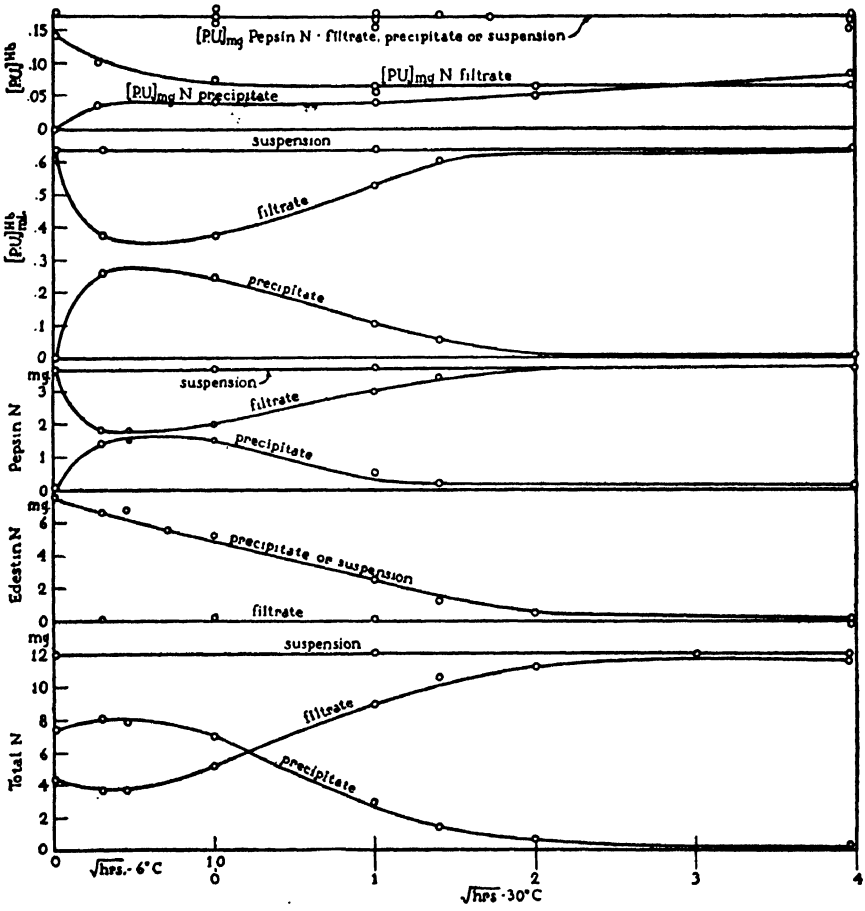

FIG. 4. Autolysis of edestin-pepsin suspensions with time.

In the first few minutes pepsin nitrogen and the corresponding amount of activity are taken up by the edestin crystals and there is a corresponding loss from the filtrate. As the reaction proceeds the quantity of precipitate becomes gradually less, the loss being due to loss of edestin nitrogen. The pepsin nitrogen and activity in the precipitate decrease, and increase to a corresponding extent in the filtrate. The specific activity of the precipitate, however, continues to increase and reaches a value of about one-half that of crystalline pepsin at the end of 24 hours. At this time there is only a very small amount of precipitate left and the solution is practically a solution of pepsin containing digested edestin. It will be noted that there is practically no change in the total activity or total pepsin nitrogen of the whole suspension at any time.

VII. Changes in Protein Nitrogen and Peptic Activity during Autolysis of Edestin-Pepsin at pH 1.5 and 35°C.

In the preceding experiment the edestin-pepsin autolyzed at about pH 4.0. If the edestin-pepsin is dissolved in hydrochloric acid the edestin is destroyed much more rapidly.

Experimental Procedure.—2 gm. crystalline edestin suspended in 10 ml. of N/10 sulfuric acid, cooled to 0°C. and poured into 40 ml. of a solution of cold, twice crystallized pepsin pH 4.0 containing 15 mg. of pepsin nitrogen per ml. Suspension stirred for 20 minutes, centrifuged and the precipitate washed with 20 ml. cold water. The precipitate stirred with 20 ml. water and titrated to pH 1.5 with N hydrochloric acid; clear solution; total volume about 35 ml. This solution was placed at 35°C. and analyzed at intervals for total protein nitrogen, pepsin nitrogen and peptic activity by the hemoglobin method.

The results of such an experiment are shown in the lower part of Fig. 5. As in the preceding experiment there is no change in the total activity of the solution as a whole nor in the quantity of pepsin nitrogen present. The total protein nitrogen decreases rapidly and is practically reduced to the value of the pepsin nitrogen in 1 hour. The specific activity calculated on the basis of total protein nitrogen, therefore, increases rapidly and soon reaches the characteristic value for pepsin itself. In the upper part of the figure is shown the result of autolysis of a solution of pure pepsin. It is evident that the course of the reaction is entirely different. In this case the activity decreases in proportion to the total protein nitrogen (pepsin nitrogen) instead of remaining constant as is the case with "edestin-pepsin" preparations. As a result the specific activity calculated on a protein

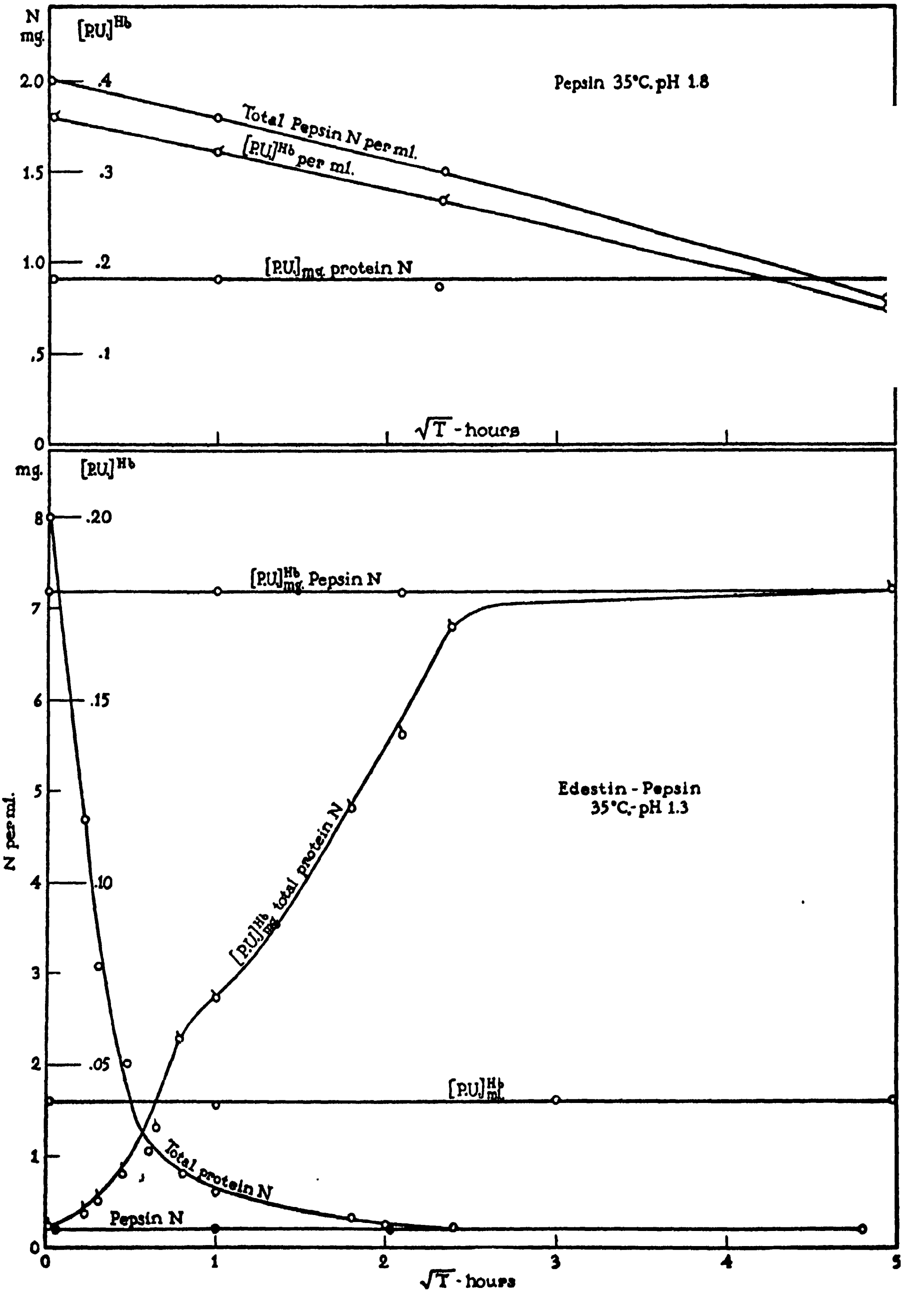

FIG. 5. Autolysis of pepsin (upper part) and of edestin-pepsin (lower part) in acid solution at 35°C.

nitrogen basis remains constant in the case of a solution of pepsin instead of increasing rapidly as in the case of a solution of "edestin-pepsin." Autolysis, therefore, serves to hydrolyze the edestin from the "edestin-pepsin" and leaves the pepsin. It will be noted that a solution of pure pepsin loses considerable activity whereas there is very little loss in activity from the "edestin-pepsin" solution. This is due to the protective effect of the products of digestion on the enzyme, probably through the formation of an additional compound of the enzyme with the products of hydrolysis of the edestin.

VIII. Preparation of Edestin-Pepsin from Edestin and Pepsin Solutions at Various pH

Experimental Procedure.—1 ml. of a 10 per cent solution of edestin in 5 per cent sodium chloride added to 10 ml. various concentrations hydrochloric acid and cooled to 0°C. 1 ml. of a solution of twice crystallized pepsin containing 1 mg. pepsin nitrogen per ml. added; precipitate appears. The suspensions kept at 0°C. for ½ hour, centrifuged and the precipitates washed once with 5 ml. cold water and dissolved in 10 ml. N/50 hydrochloric acid. The precipitate solutions were then analyzed for total nitrogen, pepsin nitrogen and peptic activity by the hemoglobin method. The edestin nitrogen is calculated as the difference between the total protein nitrogen and pepsin nitrogen. The results have been calculated to the basis of 1 ml. of the original suspension.

In the preceding experiments the edestin-pepsin was prepared by suspending edestin crystals in cold pepsin solutions. As shown by Dyckerhoff and Tewes, more active preparations may be prepared by mixing edestin solutions with pepsin solutions. The results of an experiment of this kind in which solutions were mixed at various pH are shown in Fig. 6. The figure shows that, as in the case of the absorption experiments, there is a sharp maximum at about pH 3.6. The quantity of pepsin nitrogen found in the precipitate is again equivalent to the activity of the precipitate and at the maximum amounts to 50 per cent of the precipitate. The specific activity of the precipitate at this point, therefore, is one half that of pepsin itself. As the pH becomes more and more alkaline the quantity of precipitate increases rapidly as does the per cent of edestin in the precipitate until beyond pH 5.0 the precipitate is practically all edestin.

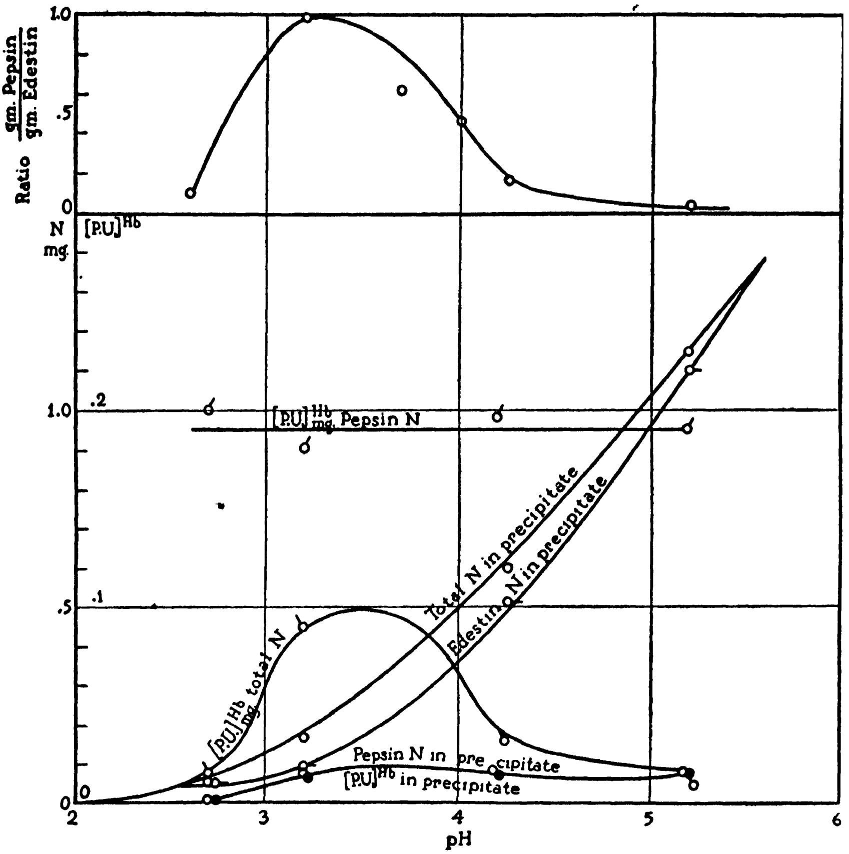

FIG. 6. Formation of edestin-pepsin from edestin and pepsin solutions at various pH.

IX. Effect of the Relative Concentrations of Pepsin and Edestin on the Composition of Edestin-Pepsin at pH 3.8 and 0°C.

Experimental Procedure.—Solutions of pepsin and edestin containing 2 mg nitrogen per ml. each, both at pH about 3.8 prepared and cooled to 0°C. A series of tubes was prepared containing 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 ml. pepsin solution and the total volume of the various tubes made up to 10 ml. with edestin solution. The tubes were kept at 0°C. for 1 hour, centrifuged and the precipitates washed with 10 ml. water and dissolved in 10 ml. N/20 hydrochloric acid and analyzed for total nitrogen, pepsin nitrogen and peptic activity by the hemoglobin method. The edestin nitrogen was calculated as the difference between total protein nitrogen and pepsin nitrogen. The results were calculated on the basis of 1 ml. of the original suspension.

The results of an experiment in which the relative quantity of pepsin and edestin were varied is shown in Fig. 7. Here the composition of the precipitate has been plotted against the composition of the original solutions. As the concentration of pepsin increases, the quantity of precipitate, its specific activity and its total activity, as well as the quantity of pepsin nitrogen, all increase and reach a maximum corresponding to equivalent amounts by weight of edestin and pepsin. As the concentration of pepsin is increased still further the quantity of the precipitate decreases but its specific activity and the proportion of pepsin in it increase slightly and then stay constant. The precipitate formed under these conditions consists of nearly three equivalents pepsin per equivalent edestin or three parts pepsin by weight per one of edestin since pepsin and edestin have the same equivalent weight. This corresponds to nearly 15 moles of pepsin per mole of edestin.

The results indicate that a definite compound is formed between the edestin and the pepsin. The isoelectric point of edestin is about pH 6.0 while that of pepsin is about 2.7 (11) so that within this range of acidity the edestin is present as a positive ion while the pepsin is present as a negative ion. According to Hitchcock (12) 0.45 gm. of edestin combines with 0.55 milliequivalents of acid. 1 gm. of edestin is slightly more, therefore, than 1 milliequivalent. 1 gm. of pepsin combines with 1.1 milliequivalent of alkali so that 1 gm. of pepsin is also slightly more than 1 milliequivalent. It is possible to determine from the titration curves of the two proteins what per cent of the protein is ionized at any pH. If this is done and it be further assumed that the positive edestin ions react with the negative pepsin ions to form a slightly soluble compound, then the position of the maximum near pH 4.0 is correctly predicted. Since the precipitate, however, has varying composition it is not possible to account for its formation on this simple basis. If a series of compounds is assumed it is possible to fit the curves quite closely but so many arbitrary constants are involved that the results are not very convincing. It is evident, however, as the writer has pointed out previously (4), that the formation of this complex is not very closely connected with the hydrolysis of the edestin since the pH corresponding to the maximum complex formation is around 4.0 while the maximum from the rate of digestion is near pH 2.0. It is probable that the reaction is quite similar to that between proteins and ordinary nucleic acids (14) and is not especially connected with the proteolytic activity of the pepsin. Unfortunately experiments cannot be carried out with inactivated pepsin since inactivated pepsin is insoluble through this range of pH.

It may be pointed out that in the preceding experiments the concentrated solutions of commercial pepsin used were highly supersaturated with regard to

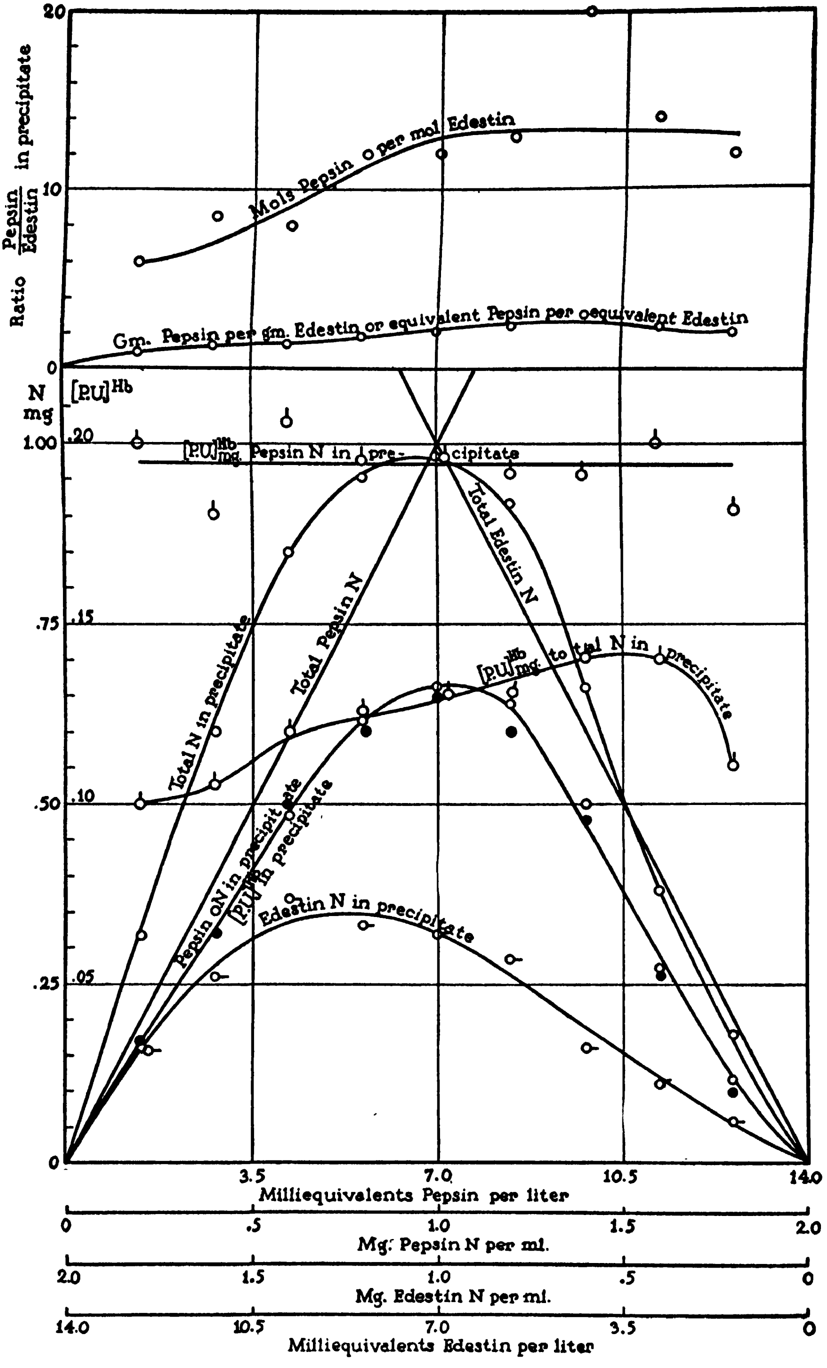

FIG. 7. Effect of the relative quantities of pepsin and edestin on the formation of edestin-pepsin at pH 4.0.

pepsin since the solubility of crystalline pepsin is quite low from pH 2.5 to pH 4.0. If concentrated solutions of crystalline pepsin had been used the results would have been entirely different since the pepsin would be precipitated from the more acid solutions even without the edestin. Commercial preparations of pepsin contain some substances, probably protein split products, which prevent the precipitation of the pepsin protein and render very highly supersaturated solutions quite stable. However, if a 10 per cent solution of Cudahy pepsin, between pH 3.0 and 4.0 is inoculated with pepsin crystals and allowed to stand at 6°C. for several weeks quite an appreciable quantity of pepsin crystallizes out.

X. Absorption of Pepsin by Melon (Cucumis) Globulin (15)

The preceding experiments were done with edestin but exactly similar results may be obtained with globulin from melon seed (*Cucumis*) as reported by Waldschmidt-Leitz and Kofrânyi (6).

TABLE VII

Absorption of Pepsin by Melon (Cucumis) Globulin

	Total nitrogen per ml.	Pepsin nitrogen per ml.	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$	$[\text{P.U.}]^{\text{Hb}}_{\text{mg. pepsin N}}$
	mg.	*mg.*		
Pepsin solution	0.067	0.056	0.009	0.16
First supernatant	0.054	0.050	0.007	0.14
Second supernatant	0.037	0.042	0.007	0.17
Third supernatant	0.066	0.024	0.005	0.20
Combined precipitates		0.030	0.005	0.165

Experimental Procedure.—100 ml. of a solution of twice crystallized pepsin containing 0.37 mg. pepsin per ml. pH about 3.5 cooled to 0°C. and 100 mg. of crystalline *Cucumis* globulin added. The suspension was stirred for 10 minutes and centrifuged. 100 mg. of globulin was added to the supernatant and the process repeated. The supernatant was again extracted with 100 mg. of globulin. The precipitates were combined, dissolved with N/50 hydrochloric acid, the solution titrated to about pH 2.0 with hydrochloric acid and made up to 100 ml. and allowed to digest at 37°C. for 4 hours. The supernatant solutions and the solution of the precipitate were then analyzed for total nitrogen, pepsin nitrogen and peptic activity by the hemoglobin method.

The results of an experiment in which a solution of crystalline pepsin was treated with successive quantities of melon globulin are shown in Table VII. As the table shows, about half the total activity is removed from the solution by the crystalline globulin and at the same time a corresponding quantity of pepsin nitrogen is also removed. The total nitrogen content of the solution decreases at first and then increases as some of the globulin dissolves and in this particular experiment it happens to be practically the same after three extractions as it was originally. This is the result which was reported by Waldschmidt-Leitz and Kofrányi from dry weight determinations and which led them to the conclusion that the active group had been removed from the pepsin protein and the inert protein left in solution. As the present experiments show, however, this is not the case. A quantity of pepsin protein equivalent to the activity removed is taken up by the foreign protein and there is no evidence that the pepsin is decomposed into an inert protein and an active group.

TABLE VIII

Absorption of Pepsin by Gelatin at Various pH

pH	4.65	4.65	4.0	3.4	3.0	2.0
Gelatin, *gm*	0	2.5	1.0	1.0	1.0	1.0
Pepsin solution, *ml*	0.5	0.5	1.0	1.0	1.0	1.0
$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$ supernatant	0.0074	0.0029	0.0071	0.0091	0.0083	0.013
$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$ gelatin		0.05	0.034	0.024	0.022	0.016
Pepsin nitrogen in gelatin, *mg*	0	0.25	0.16	0.13	0.10	0.078
$[\text{P. U.}]^{\text{Hb}}_{\text{mg.}}$ pepsin nitrogen in gelatin		0.20	0.21	0.185	0.22	0.20
Ratio $\frac{[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}\ \text{gelatin}}{[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}\ \text{supernatant}}$		17.0	5.0	2.6	2.6	1.2

XI. Absorption of Pepsin by Gelatin at Various pH

Experimental Procedure.—A series of suspensions of powdered isoelectric gelatin in various concentrations of hydrochloric acid, total volume 50 ml. was prepared and cooled to 6°C. for ½ hour. 1 ml. of dilute crystalline pepsin solution was added and the suspension stirred for ½ hour and filtered. The gelatin precipitates were melted at 37°C. and the solutions analyzed for pepsin nitrogen by precipitation with hot trichloracetic acid and peptic activity by the hemoglobin method.

In the experiments with gelatin reported previously (4), the activity alone was followed and no determinations were made of the changes in pepsin nitrogen since at that time it was not known that the activity was a property of the pepsin protein. The experiments have, therefore, been repeated and determinations made of the pepsin protein in the gelatin as well as of the activity. The results of the experiment are shown in Table VIII. As in the experiments with edestin, the quantity of pepsin protein taken up by the gelatin is just equivalent to the activity found in the gelatin so that in this case also the pepsin protein itself is taken up by the foreign protein. It will be noted that much more is taken up with isoelectric gelatin than by acid gelatin. This peculiarity was noted before (4) and was found to be due to surface adsorption on isoelectric gelatin whereas with acid gelatin the quantity taken up was independent of the surface.

XII. Inactivation of Pepsin by Alkali in the Presence of Edestin

Warburg and Christian (13) have found that the respiratory ferment decomposes into a protein and a non-protein fraction when the protein is denatured. Hemoglobin also possesses the same peculiarity. It seemed possible, therefore, if the pepsin were denatured by alkali in the presence of edestin, which is not affected by dilute alkali, that the active group might leave the pepsin and become attached to the edestin. In this case it would be expected that a loss in activity of pepsin solutions containing edestin in alkali would be less than the loss in activity of pure pepsin solutions at the same pH. This, however, is not the case as shown in Table IX. Evidently either pepsin does not decompose into two parts when the pepsin protein is denatured or if it does the prosthetic group has no activity under these conditions and edestin cannot replace the pepsin protein. The fact that active pepsin can be prepared from pepsin denatured by alkali indicates that splitting of the molecule does not occur although the yield of active pepsin is so small as to render this argument more or less inconclusive.

TABLE IX

Inactivation of Pepsin by Alkali in the Presence of Edestin

pH		5.0	5.6	8.0
$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$	"Edestin pepsin"	0.0074	0.0072	0.001
	Pepsin solution	0.0075	0.0073	0.0008

Experimental Procedure.—100 mg. of edestin suspended in 10 ml. of water and 1 ml. dilute crystalline pepsin solution added. Increasing amounts of alkali added to a series of these tubes and the suspension allowed to stand at 25°C. for 10 minutes. Control series without edestin prepared in the same way. The pepsin activity determined by the hemoglobin method.

XIII. Spheroidal Pepsin

Dyckerhoff and Tewes found that an active precipitate could be obtained from autolyzed edestin-pepsin or from partly autolyzed pepsin solutions which appeared in the form of spherical, highly refractile granules which they called "*kugeln*" pepsin. These spheroids are characteristic of proteins which are not sufficiently purified or which appear under unfavorable conditions for crystallization. The spheroids obtained from autolyzed "edestin-pepsin" solutions consist, as described under Experiment 2, almost entirely of pure pepsin with varying amounts of some form of non-protein nitrogen. The same precipitate may be obtained from pepsin solutions which have been allowed to stand and which are too acid and too dilute for crystallization to take place readily. They may be obtained as characteristic pepsin crystals by dissolving with alkali, precipitating rapidly in the amorphous form with sulfuric acid and crystallizing as usual. The results of such an experiment are shown in Table X.

TABLE X

Spheroids

Fraction No		(1) Spheroids	(5) Crystals plus amorphous	(6) Filtrate	Original twice crystallized pepsin
N/ml., *mg.*	Total	12.0	0.60	4.1	
	Protein	9.5	0.45	3.7	
$[\text{P. U.}]^{\text{Hb}}$	/ml.	1.8	0.066	0.71	
	/mg. N	0.15	0.11	0.17	0.17
	/mg. protein N	0.19	0.15	0.19	0.19
$[\text{P. U.}]^{\text{Gel. V}^-}$	/ml.	160	20	56	
	/mg. N	13.3	33	13.5	10
	/mg. protein N	17	44	15	12
$[\text{P. U.}]^{\text{Cas V}^-}$	/ml.	11,500	380	3,800	
	/mg. N	960	630	930	1,000
	/mg. protein N	1,200	850	1,030	1,100

Experimental Procedure.—15 gm. twice crystallized pepsin filter cake dissolved in 200 ml. water at 45°C. and 5 ml. N/2 sodium hydroxide added (clear solution); 10 ml. N/2 sulfuric acid added, precipitate forms (pH 2.4) and suspension left at 6°C. for 24 hours. Precipitate small spheroids, filter, 10 gm. cake (1). 50 ml. water and 4 ml. N/2 sodium hydroxide added to (1) (clear solution). 3 ml. N/2 sulfuric acid added, slight precipitate, (spheroids), suspension cooled slowly to 20°C. Filter, precipitate (2) 5 gm. (2) spheroids dissolved in 15 ml. water and 1 ml. N/2 sodium hydroxide added (clear solution) (3). (3) titrated to pH 3.0 with sulfuric acid and cooled to 6°C., amorphous precipitate, filter and precipitate dissolved at 45°C. with sodium hydroxide. Solution titrated to pH 3.5 with sulfuric acid, cooled slowly to 6°C. for 4 days. Precipitate of poor crystals formed. Filter and precipitate dissolved in N/50 pH 5.0 acetate (5). Filtrate (6).

The activity was determined by several methods. The specific activity calculated on the basis of protein nitrogen is constant for the hemoglobin and casein method in all the fractions but is slightly low as calculated on the basis of total nitrogen. The specific activity, as determined by the gelatin viscosity, is high as is always the case when a small fraction is precipitated from incompletely purified pepsin solutions since the gelatinase is concentrated in such precipitates.

The analytical work reported in this paper was done by Mr. N. Wuest.

SUMMARY

Crystalline proteins, such as edestin or melon globulin, remove pepsin from solution. The pepsin protein is taken up as such and the quantity of protein taken up by the foreign protein is just equivalent to the peptic activity found in the complex. The formation of the complex depends on the pH and is at a maximum at pH 4.0.

An insoluble complex is formed and precipitates when pepsin and edestin solutions are mixed and the maximum precipitation is also at pH 4.0. The composition of the precipitate varies with the relative quantity of pepsin and edestin. It contains a maximum quantity of pepsin when the ratio of pepsin to edestin is about 2 to 1. This complex may consist of 75 per cent pepsin and have three-quarters of the activity of crystalline pepsin itself. The pepsin may be extracted from the complex by washing with cold N/4 sulfuric acid. If the complex is dissolved in acid solution at about pH 2.0 the foreign protein is rapidly digested and the pepsin protein is left and may be isolated.

The pepsin protein may be identified by its tyrosine plus tryptophane content, basic nitrogen content, crystalline form and specific activity.

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ELECTRICAL RESPONSES FROM THE LATERAL-LINE NERVES OF FISHES

IV. THE REPETITIVE DISCHARGE*

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I

The electrical responses from lateral-line nerves of trout and catfish have been described in earlier papers (Hoagland, 1932–33 *a*, *b*, 1933–34). These nerves normally appear to be in a state of vigorous activity, discharging impulses in the absence of externally applied stimuli. Modification of the spontaneous discharge during stimulation of the sense cells (neuromasts) served as an index of the receptivity of these cells to mechanical stimuli and of the effects of temperature.

In addition to nerve impulses initiated from the receptor cells a discharge of impulses sometimes arises spontaneously from damaged nerve endings of the lateral-line fibers. This discharge resembles that from the neuromasts. Discharges from both of these sources, especially in trout lateral-line nerves, may become synchronized. Properties of this synchronized response are quite definite and may mimic those of a single functional unit. The present paper discusses the discharge from the neuromasts and that due to injury, as well as the synchronized discharge. The possible pace-making process involved in the synchronization is also considered.

The nature of mechanisms underlying the repetitive discharge of nerve impulses from tissues maintaining states of excitation is an important problem for nervous system dynamics. Contributions to its development are desirable for the advancement of a general physiol-

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ogy of the receptors and are also significant in the investigation of central nervous phenomena encountered in the study of spinal reflexes, such typically as excitation, inhibition, occlusion, facilitation, and after discharge.

Sherrington has accounted for these phenomena in terms of central excitatory and inhibitory states which may sum algebraically, essentially as specific chemical entities, facilitating or inhibiting the passage of impulses across synapses (*cf.* Creed, Denny-Brown, Eccles, Liddell, and Sherrington, 1932).

Adrian (1928, 1932) and other workers have shown great differences in the abilities of receptors to discharge nerve impulses in response to constant stimulation. Superficial tactile receptors adapt very rapidly, giving only a few impulses in response to a constantly applied pressure (Adrian, Cattell, and Hoagland, 1931; Cattell and Hoagland, 1931; Hoagland, 1932-33 *c*) while muscle spindles adapt much more slowly, initiating impulses in their nerve fibers for a minute or more during a constant tension on the muscle (Matthews, 1931 *a*, *b*). The spontaneously discharging lateral-line receptors seem to be end-organs which essentially show no adaptation. It is as if normal metabolic processes occurring in these receptors were capable of maintaining an excitatory state such that nerve impulses are initiated at a frequency determined by the excitatory process. A somewhat similar state of affairs appears to be maintained in the respiratory centers of animals, since Adrian (1931) has shown that the brain stem of the goldfish when removed from the body undergoes rhythmic and "spontaneous" changes of potential at a frequency corresponding to the normal opercular breathing rhythm. Adrian (1931) has also shown that the thoracic and abdominal ganglia of the beetle *Dytiscus marginalis* periodically show variations in potential independently of afferent control. The electrical variations produce corresponding bursts of impulses from nerves connected with the ganglia. A similar rhythmicity of the respiratory center of mammals and its fundamental independence of afferent control has been demonstrated by Bronk and Ferguson (1933). Lillie (1929) has been able to make a model of a receptor with its attached nerve fiber from the well known iron wire, nitric acid system. By covering a part of the wire with glass tubing he so limited the diffusion of acid, and hence the recovery process, of the glass-sheathed wire that continuous oxidation of the iron took place within the tube. This continuous action was found to set up rhythmic discharges of impulses over the acid-immersed wire outside of the tube as fast as it recovered from the refractory period following each impulse.

Adrian (1930) described types of repetitive and rhythmic discharges of impulses arising from injured mammalian nerve fibers. He found that when several injured fibers were discharging repetitively the impulses may become synchronized, and he concluded that the synchronization is probably due to the superimposed stimulating effect of action currents of one fiber on its neighbors. The persistently discharging fibers appeared to be small sensory fibers which, for the most part, are distributed to blood vessels and fascia. He concluded that the persistent discharge arises from the injured fibers, due to permanent depolarization of the injured portions acting as a region of heightened excitability for the intact part of the fiber and he pointed out the similarity between the injury discharge and that set up from receptors by stimuli which may depolarize them. Certain aspects of the "spontaneous" activity of the lateral-line system illuminate several of the general problems of persistent activity suggested by the foregoing discussion.

The experimental procedure consists in baring the lateral-line nerve on one side a centimeter behind the head and dissecting it free for 1 or 2 cm. It is then tied, cut cephalad, and the freed end is drawn across silver-silver chloride electrodes connected to the recording system. The action potentials of the nerve are amplified and recorded by means of a Matthews oscillograph used in conjunction with a camera and a standing wave screen. A loud speaker makes the amplified action potentials audible.

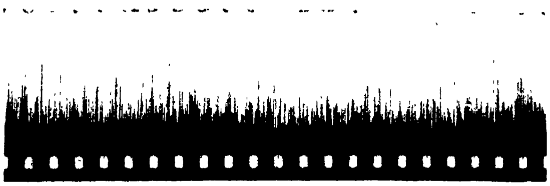
II

The continuous spontaneous action of the lateral-line receptors in fishes is dependent on an intact circulation. With decapitated catfish the spontaneous discharge may show no sign of failure for an hour or more, if the skin is kept immersed in water and if the nerve and incision are well moistened with Ringer's solution. With decapitated trout, failure of the response generally occurs in about 15 minutes. These facts are consistent with the general dependence of the two forms on oxygen requirements. Catfish can live in extremely foul water and even remain alive in air for several hours; in fact, they are often wrapped in moistened paper and shipped in the air from fish hatcheries to streams and lakes. Trout, on the other hand, die rapidly if removed from the water or if kept for appreciable lengths of time in foul water. If one immobilizes a trout by transecting its cord without cutting the larger blood vessels the preparation lasts much longer, even though opercular breathing is not evidenced, as it generally is in corresponding preparations of catfish. By recording the heart beat with a second pair of electrodes connected through a switch to the recording system, one finds that impulses are given off from the neuromasts as long as the heart continues to beat. With four such trout preparations in which care was taken to keep the nerve moistened with Ringer's solution (the flank was under water) the response continued for 57, 62, 48, and 69 minutes in each of the four cases, failing in from 10 to 20 minutes after cessation of the electrocardiogram. Owing to the great difficulty of immobilizing the fish without destroying its circulation, only four of these stable trout preparations have been obtained. In addition to the usual rapid failure of response from the trout receptors and the great density of the discharge which makes unreliable the counting of impulses from the records, a further difficulty arises rendering quantitative analysis of the responses from the receptors unreliable. An injury discharge from the nerve often develops which may resemble and mask that from the neuromasts. This injury discharge developed in 43 of some 60 trout nerves tested. It sometimes occurs in catfish but in only about 20 per cent of the cases.

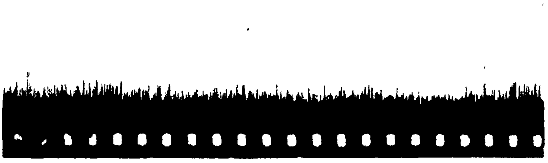
The injury discharge is of considerable interest. It is similar in many respects to that described by Adrian (1930) from injured mammalian nerve fibers. That the neuromasts discharge impulses independently of injury may be demonstrated by slicing along the flank between the region of exit of the nerve to the electrodes and the tail. In the trout as in the catfish this procedure causes a decline in the activity depending upon the number of uninjured neuromasts which have been thrown out of action posterior to the cut.

Fig. 1 shows the decline in response from a trout preparation which showed no injury discharge (*cf.* also Hoagland, 1932–33 *b*, Figs. 1 to 3). Experiments of this kind must be done rapidly with trout since a few minutes after the initial operation of freeing the nerve, the impulses due to injury may begin and increase in volume. At the end of about 10 minutes from the beginning of the experiment the entire discharge of impulses begins to fail with trout in which hemorrhage has been sufficient to interfere with the circulation, and the nerve is generally silent within 10 to 15 minutes of the start of the experiment. In three of the four cases in which the circulation was intact the frequency of the "spontaneous" discharge increased for some 15 minutes probably due to injury effects and then returned to a basal constant frequency which was clearly due to the activity of the neuromasts since slicing through the lateral-line produced a decline of response due to the removal of neuromasts caudal to the fresh cut.

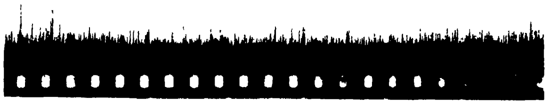
110 Receptor Groups



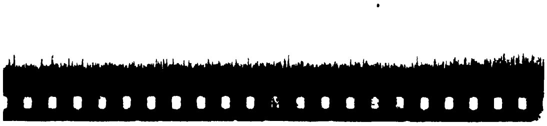
60 Receptor Groups



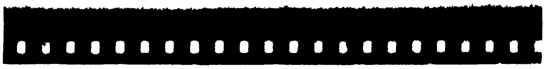
15 Receptor Groups



3 Receptor Groups



No Receptor Groups



⟷ 0.1 second

Fig. 1. Spontaneous discharge of impulses from the lateral-line nerve of a trout as a function of the number of active neuromast groups. No injury discharge occurred in this preparation.

This procedure tests the occurrence of injury discharge since in cases in which no injury effect is manifested, cutting the connection of the nerve with its neuromasts or crushing it between the body and electrodes completely stops all impulses. When the injury discharge occurs the nerve may continue firing, even after cutting or crushing it between the electrodes and the body of the fish.

A curious feature common to the discharge from the neuromasts and the injury discharge is the tendency for the impulses in the fibers to become synchronized. Fig. 2 shows a typical injury discharge from a trout lateral-line nerve, produced by slowly pulling the nerve and progressively breaking all connections with the neuromasts without breaking the main nerve trunk. The discharge, at first asynchronous, becomes synchronized spontaneously after a few minutes and conspicuously manifests itself through the loud speaker—the roar of impulses becomes a musical note. In Fig. 2 the series of photographs shows an increase in frequency of the synchronous discharge up to about the 10th minute followed by a decline for 2 minutes ending in an asynchronous discharge. In a few preparations the synchronized frequency waxes and wanes, producing a siren effect from the loud speaker, each rise and decline of pitch taking from 30 seconds to a minute. In nerves in which the synchronized response involves both injury effects and receptor activity, progressive cutting away of neuromasts, which produces a decline in the number of active fibers, has no effect on the frequency of the beat, but usually lowers its amplitude owing to the removal of some of the fibers, impulses from which were summing to give the synchronized discharge. When the injury discharge is well developed cutting away neuromasts may have little effect on the response, the frequency may remain unchanged, and the amplitude may be only slightly reduced even by separating from the body a fragment of nerve on the electrodes. Fig. 3*a* shows records of a synchronized discharge at a frequency of approximately 400 per second produced by 102 neuromast groups. Fig. 3*b* shows a residual injury discharge after cutting the nerve between the body and the electrodes. The frequency is still 400 per second, but the amplitude is greatly reduced owing to the inactivation of fibers carrying the discharge from the neuromasts.

Cooling the trout receptors produces, as in the catfish, a decline

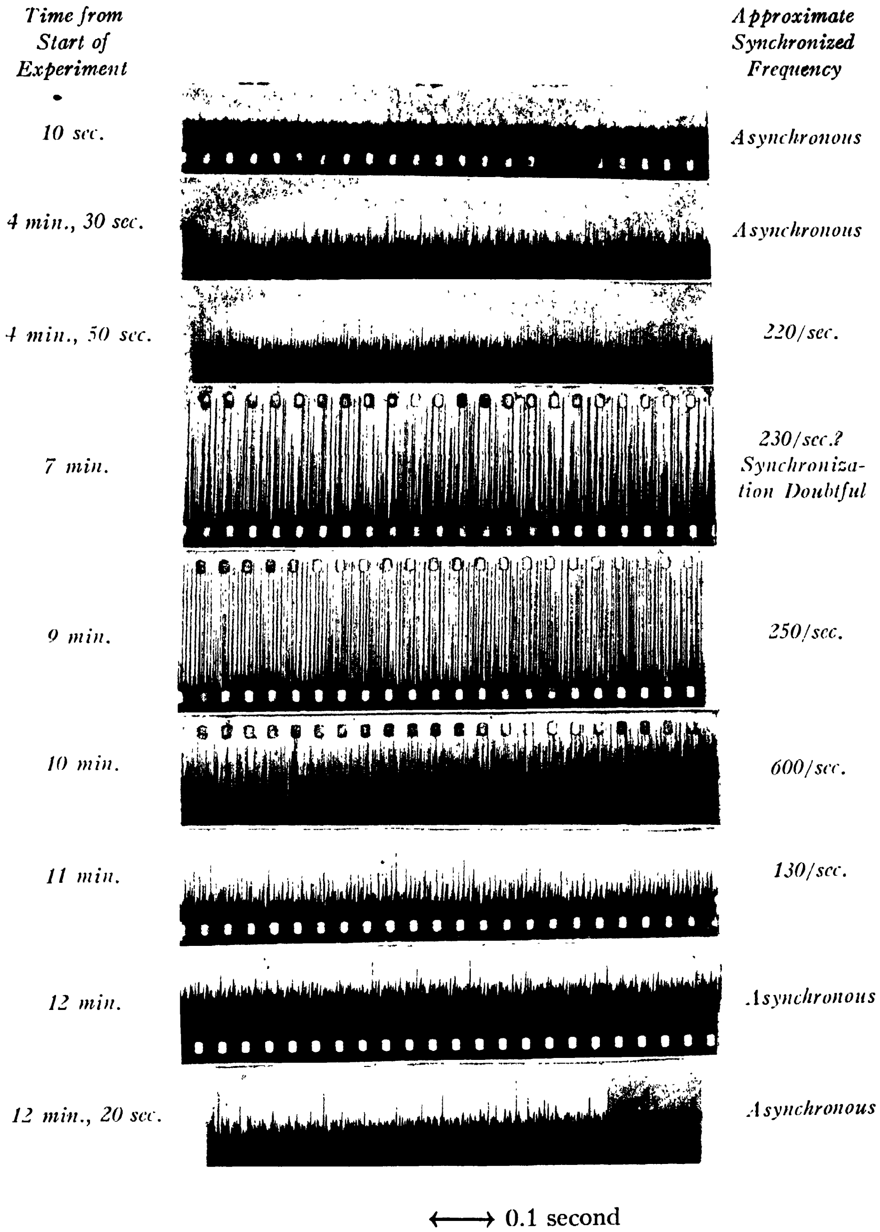


FIG. 2. Injury discharge from a trout lateral-line nerve produced by pulling the nerve and breaking connections with the neuromasts. The discharge was random for the first 4 minutes and increased in intensity becoming synchronized during the 4th minute at a frequency of 120 per second. The frequency of the synchronous discharge increased up to about 10 minutes to a maximum of 600 per second, then declined, becoming asynchronous again at 120 rhythms per second and finally ceasing altogether at about 13 minutes. Temperature 21°C.

in the frequency of the discharge provided the response is asynchronous. If it is synchronous, cooling reduces the amplitude of the response without affecting the frequency, an effect similar to that of surgically removing the neuromasts, due, probably, to the inactivation of some of the receptors, since the previous work (Hoagland, 1932–33*b*) has shown that the neuromasts of catfish have diverse temperature thresholds for activation. The removal of fibers contributing to the synchronous discharge reduces the amplitude of the response by

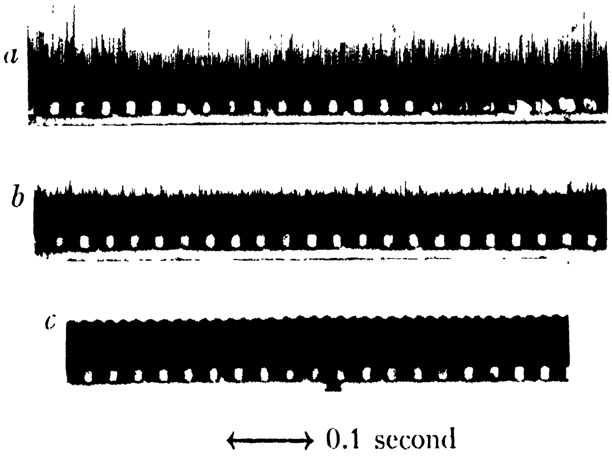


FIG. 3. (*a*) Synchronized discharge with 100 contributing neuromast groups.

(*b*) Residual injury discharge at same frequency as in (*a*) immediately after cutting the nerve at its entrance to the body.

(*c*) Base line after crushing the nerve at the electrodes. The ripples in the base line are due to imperfect shielding from alternating current disturbances.

reducing the summed effect of the impulses. When the response is asynchronous, lowering the temperature produces a decline in the frequency of the discharge but to a less marked degree than in catfish. Quantitative data relating temperature and frequency have been difficult to obtain with trout and are unreliable owing to the rapid failure of most preparations and to the complications of the injury discharge.

In a previous paper (Hoagland, 1933–34) it was pointed out that compression of tissues surrounding the lateral-line receptors in trout and catfish initiates impulses against the background of spontaneous activity. The fibers activated by this stimulation give larger action potentials than those normally contributing to the repetitive impulse discharge initiated by the neuromasts. Fig. 4*a* shows responses produced by stroking the caudal flank of a trout over the lateral-line canal after cutting away all but three spontaneously firing neuromasts (*cf.* Hoagland, 1933–34). The response to stroking is indicated by the bursts of impulses. Fig. 4*b* shows the effect of stroking the same preparation some minutes later after the response had spontaneously become synchronized at a frequency of 400 per second. The discharge from the tactile receptors now appears to be phasic, although the stimulation—stroking with a feather tip—is the same as that used in producing Fig. 4*a*. It is as if a periodicity had been set up in the nerve and only impulses coinciding with the phasic relations of the spontaneous periodic discharge could be transmitted along the fibers. Since the impulses are carried in fibers not contributing to the spontaneous discharge, this phenomenon cannot be accounted for on the assumption of limitation of conduction due to the absolute refractory period of these individual fibers. The alternating refrac-

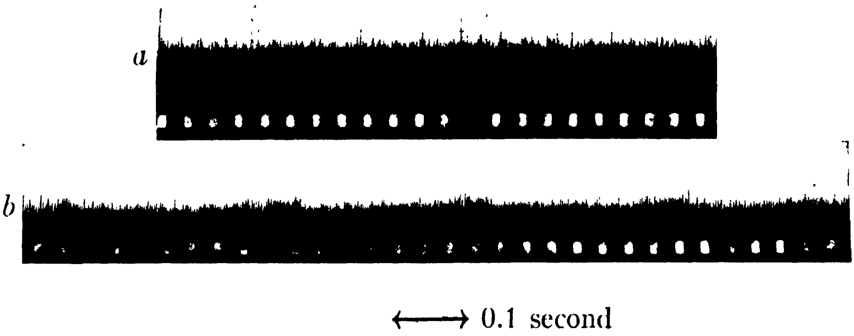


FIG. 4. (*a*) Effect of stroking the skin above the lateral-line canal in a trout. Two bursts of impulses are recorded against the "spontaneous" background. Three groups of neuromasts were contributing to the spontaneous response.

(*b*) Effect of stroking the same preparation after the spontaneous response had become synchronized. Effects of four strokes are shown. The impulses in the tactile fibers sum to increase the amplitude of the response without breaking the spontaneous rhythm of 400 per second.

tory and excitatory phases must occur across the whole nerve alternately blocking and facilitating the passage of impulses from any fiber which may be brought into action by the external stimulus. From Fig. 4*a* it is clear that the stimulus shows no ability to excite fibers at frequencies high enough to permit inhibition by the refractory period of the individual fiber. Since the oscillograph has a natural period of about 6500 per second, synchronization of tactile impulses with the spontaneous discharge cannot be accounted for in terms of an instrumental periodicity.

Electrical spread of action currents beyond the confines of the fibers in which they occur might produce alternating fluctuations of excitability in all the fibers of a nerve trunk including the unstimulated ones. When these latter fibers become active through stimulation they could only conduct at periods coinciding with the periods of excitability produced by the activity of the other fibers which are spontaneously active.

The last suggestion obviates the necessity of any localized pace maker for the phasic response. Anesthetization, or crushing of the freed, cut end of nerve distal to the electrodes, seldom makes any difference whatever in the response. Very occasionally some diminution of the discharge results from this treatment but this may be due to movement of the nerve on the electrodes. The only damaged region, therefore, common to all of the nerve fibers seems to have little or nothing to do with the synchronous discharge. The groups of neuromasts are distributed along the course of the nerve and since they can be removed either surgically, or functionally by lowering the temperature, without affecting the synchronous rhythm, no single position along the course of the nerve may be regarded as a pace maker region. In some preparations when the injury discharge occurs all the neuromasts may be cut away by severing the nerve between the body and the electrodes, and the only effect on the synchronous response is to decrease its amplitude as in Fig. 3 without altering its frequency. These facts indicate that synchronization is independent not only of the neuromasts but also of any one anatomically fixed place along the lateral-line nerve.

The injury discharge takes origin, primarily, from the cut and broken endings of the connections to the neuromasts in the region of the exposed length of nerve which has been removed from the side for recording purposes. This operation necessitates the breaking of connection with some dozen or more groups of sense cells. Since cutting the nerve free from the fish where it leaves the body has little effect on the response when the injury discharge is well developed, and since crushing the severed ends of the nerve also is generally without effect, it appears that the broken connections with the neuromasts may be the source of the injury discharge. This is supported by the fact that the magnitude of the discharge is progressively reduced by successively pinching the length of nerve severed from the fish along its course between the body and the input electrode. The fact that pulling the nerve supplying the sense cells and breaking connections with neuromasts generally produces violent injury discharges further supports the view that the discharge arises from the depolarized broken ends of fibers supplying the sense cells. These facts indicate that stimulation of the injured fibers is a result of the permanent depolarization due to the injury, a conclusion in agreement with that reached by Adrian in accounting for the injury discharge from mammalian fibers. Since the injury discharge normally resembles so completely the spontaneous discharge initiated by the sense cells it is possible that these cells normally excite the nerve terminations by producing a relatively permanent region of depolarization at the fiber terminations as a result of their normal metabolic activity. The dependence of the response from the neuromasts on the circulation supports this suggestion. The fact that the non-spontaneously discharging tactile receptors fail to respond in all preparations when the spontaneous discharge set up by the neuromasts fails, suggests the dependence of excitabilities of both groups of receptors on essentially similar steady metabolic conditions.

III

Adrian (1930) found that rhythmicity of impulses discharged from injured mammalian nerve fibers at body temperature does not occur at frequencies of less than 150 per second. When the frequency declines below that the discharge becomes aperiodic. This critical minimum frequency of discharge was accounted for by Adrian in terms of the curve describing the strength of stimulus required to excite a nerve during various stages of its recovery period. This curve is hyperbolic and indicates that the greater a constant stimulus is above the threshold amount necessary to excite the fully recovered nerve, the greater will be the frequency of discharge which it may produce from the nerve during the relative refractory period. A constant stimulus of exactly the threshold amount necessary to excite fully recovered nerve would be able, theoretically, to initiate only one impulse since it would take infinitely long for the nerve to recover to the threshold asymptote where this stimulus would again be effective. The critical frequency of 150 per second implies, therefore, a recovery period of 6.7 σ for the mammalian fibers and this agrees well with recovery periods of 5 to 10 σ measured in motor fibers by Adrian and Olmsted, and Erlanger, Bishop, and Gasser.

In the lateral-line nerve of the trout the critical minimum frequency is 120 rhythms per second corresponding to a recovery period of 8.3 σ. The maximum frequency of 600 per second indicates either that the fibers are being discharged by a strong constant stimulus very early in their relative refractory periods or else that different fibers may respond in successive rhythms of the discharge. If the fibers, for example, were able to respond at a maximum frequency of only 300 per second, a rhythm of 600 per second might be obtained by having two groups of fibers alternately firing at 300 per second. A mechanism of this kind has been proposed by Troland (1929) to account for the conduction of high auditory frequencies. The maximum frequency of 600 impulses per second recorded in Fig. 2 is higher than that usually associated with responses from single fibers in cold blooded vertebrates. It is, nevertheless, possible that this rhythm is produced by the same group of fibers discharging at this frequency.

Many of the experimental facts described above as well as certain aspects of the injury discharge presented by Adrian suggest the notion of chemical mechanisms underlying the repetitive discharges. The rise of the injury discharge from the lateral-line nerve (*cf.* Fig. 2) begins some seconds after breaking connection with the neuromasts, builds up to a maximum in about 10 minutes, and then declines to zero during the next 5 minutes. The rise and decline of frequency of impulses could be accounted for if the frequency were proportional to the concentration of a substance (*B*), produced as an intermediate compound in consecutive reactions of the type

$$A \rightarrow B \rightarrow C$$

Adrian found that the injury discharge from mammalian fibers is reduced or stopped by irrigation with Ringer's solution independently of variations in the composition of the solution. He found that the discharge, silenced by washing with Ringer's, was renewed in the presence of serum. Crushed tissue extracts in contact with the nerve did not produce nerve impulses and he concluded that a substance supplied by the circulation was essential for the production of the injury discharge.

The dependence of the discharge from the fish neuromasts for periods of greater than 15 minutes on the integrity of the circulation indicates that this normal effect depends on something continuously supplied by the serum. This may possibly be the *A* substance of the above paradigm which breaks down continuously to the chemically stimulating *B* substance in the sensory cells. Rupture of connections with the neuromasts would remove the broken endings from further supply of *A* by the circulation but might leave a fixed amount of *A* in part of the nerve which, on mixing with enzymes released from the broken tissue, would then decompose to form *B*, the source of the impulses due to injury. In this way the curious cycle of rise and decline of the injury discharge might conceivably be accounted for.

There are, of course, other possible alternatives. For example, the circulation may act to remove substances normally inhibiting a process tending to produce continuous excitations.

SUMMARY

The spontaneous discharge of impulses from the lateral-line nerves of trout and catfish has been examined.

1. Broken endings of nerve fibers supplying receptors of the lateral-lines of trout and catfish may be the source of a repetitive discharge of nerve impulses.

2. This injury discharge occurs more frequently in trout and may

mask the spontaneous discharge from the receptor cells. Experiments indicate that the latter discharge is not the result of injury.

3. The injury discharge ceases in from 10 to 15 minutes. The spontaneous receptor discharge in trout may continue for an hour if the circulation remains intact. The receptor response also fails in from 10 to 15 minutes after failure of the circulation.

4. The receptor discharge, the injury discharge, or the summed discharges frequently become synchronized. The excitability of the fibers of the nerve trunk appears to vary synchronously, so that nerve impulses initiated in fibers from tactile receptors not contributing to the spontaneous discharge can be conducted only during the part of the cycle occupied by the spontaneous discharge.

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THE THEORY OF DIFFUSION IN CELL MODELS

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I

INTRODUCTION

Osterhout and Stanley[1] have recently described some interesting experiments on diffusion between two aqueous phases separated by a non-aqueous phase. These experiments were modeled after living cells and duplicated the power of some cells to concentrate preferentially certain chemical substances. Furthermore, the volume increase shown by one of the aqueous phases in the model is somewhat analogous to the growth of living cells. In a model in which water and a salt are the only substances whose diffusion need be considered Osterhout[2] has shown that the results are in qualitative accord with the kinetics which characterize two consecutive monomolecular reactions. It seems to the author, however, that the mechanism more nearly corresponds to that of two simultaneous and mutually dependent processes. Consequently it is the purpose of this paper to formulate and solve the simultaneous differential equations which probably describe the model. These equations will be derived starting with the simple but fundamental laws which describe diffusion processes in general.

Jacobs[3] has recently formulated the simultaneous differential equations which describe the relation between cell volume and penetration of a solute. These equations, though similar to the ones which are derived in this paper, are somewhat more difficult to solve and the desired time curves are not obtained by direct integration.

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[1] Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931–32, **15**, 667.

[2] Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529.

[3] Jacobs, M. H., *J. Cellular and Comp. Physiol.*, 1933, **3**, 29.

II

Description of the Experiment

Osterhout[2] has given a detailed description of the experiment which is to be treated theoretically in this paper. He has also included an excellent discussion of the physical processes which are involved and the justification for certain assumptions which it will be necessary to make in the development of the theory. Therefore only a brief discussion of the experiment will be given in the following paragraphs.

The aqueous layer A (Fig. 1) consists of water saturated with a weak acid, HG, and contains the potassium salt of this acid, KG, at a concentration of 0.05 normal. The solution in A is continuously replaced during the experiment so as to maintain a constant concentration of KG in this region. The non-aqueous layer B consists of the weak acid HG which is but slightly miscible with water and which is initially saturated with the latter. This phase separates A from a second aqueous layer C which is saturated with HG and through which CO_2 is continuously bubbled at atmospheric pressure. All three phases are stirred so that the diffusion which occurs takes place in the layers at the phase boundaries a and b which are not affected by the stirring. As Osterhout has pointed out the high viscosity of the non-aqueous medium makes it probable that the unstirred layers are much thicker in this phase than in the outer aqueous layers. It will therefore be assumed that the only gradients of composition which occur are in the layers aa' and bb' (Fig. 1). The mobilities of the diffusing constituents are much lower in the non-aqueous phase[4] than in the aqueous phases, an additional fact which makes it probable that the constants for the diffusion processes are determined largely by the characteristics of phase B.

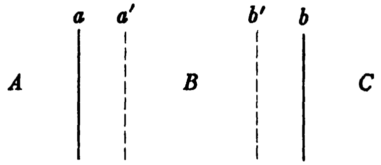

FIG. 1

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[4] Osterhout, W. J. V., Kamerling, S. E., and Stanley, W. M., *J. Gen. Physiol.*, 1933–34, **17**, in press, (Kinetics. VI).

The two substances whose simultaneous diffusion through B is significant are KG and H_2O. The force which causes the diffusion of KG, for example, from A to C is the gradient of chemical potential for this substance which exists between A and C due to the difference in the concentration of this substance in the two layers. The chemical potential μ_i, of any given molecular species i may be defined by means of the equations[5]

$$\mu_i = \mu_i^0 + RT \ln a_i \tag{2.1}$$

$$= \mu_i^0 + RT \ln \gamma_i N_i \tag{2.2}$$

In these equations μ_i^0 is the chemical potential in some arbitrarily chosen standard state, R the gas constant, T the absolute temperature, a_i the activity, γ_i the activity coefficient, and N_i the concentration of the i-th constituent. The concentration scale best adapted for the treatment of the present problem will be considered in the following section.

The KG diffuses from A in which the concentration, and hence the chemical potential, of this substance is maintained at a constant value, through B into C where its concentration is initially zero and where the following reaction occurs.

$$\mathrm{K}G + \mathrm{H_2CO_3} = \mathrm{KHCO_3} + \mathrm{H}G \tag{2.3}$$

The mass action expression for this reaction is

$$a_{\mathrm{H}G} \cdot a_{\mathrm{KHCO_3}} = a_{\mathrm{K}G} \cdot a_{\mathrm{H_2CO_3}} \cdot \text{constant} \tag{2.4}$$

Since the partial pressure of CO_2 is constant and since the activity of HG in C is maintained at a substantially constant value by contact with the non-aqueous phase in which HG predominates, equation (2.4) may be simplified to

$$a_{\mathrm{KHCO_3}} = K \cdot a_{\mathrm{K}G}$$

K in this expression being a constant.

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[5] Lewis, G. N., and Randall, M., Thermodynamics, New York, McGraw-Hill Book Co., Inc., 1923, 254. See also Gibbs, J. W., Collected works, Longmans, Green and Co., New York, 1928, 1, 92.

If the salt activities in this expression are replaced by the products of the ion constituent activities according to the convention of Lewis and Randall (p. 326 of Footnote 5)

$$a_{K^+} \cdot a_{HCO_3^-} = K\, a_{K^+} \cdot a_{G^-}$$

or

$$a_{HCO_3^-} = K\, a_{G^-} \tag{2.5}$$

Since the activity coefficients which may be inserted into equation (2.5) in order to convert the activities to concentrations always occur as a ratio, it follows from the principle of the ionic strength that a very close approximation to (2.5) will be obtained by placing

$$N_{KHCO_3} = K \cdot N_{KG} \tag{2.6}$$

Since H_2CO_3 is a much stronger acid than H*G* the constant of equation (2.6) has a value much greater than unity and most of the K*G* which diffuses into *C* is converted into $KHCO_3$.

The back diffusion of $KHCO_3$ from *C* to *A* may be neglected due to the very slight solubility of this substance in the non-aqueous phase. CO_2 appears to be able to diffuse from *C* to *A*. This does not occur to any appreciable extent in the model, however, and may therefore be neglected. It is possible that some CO_2 does diffuse across the phase boundary *b* but if the reaction represented by equation (2.3) occurs in phase *B* the $KHCO_3$ thus formed would be immediately extracted by the aqueous layer *C*, thus compensating for the diffusion of CO_2.

Whereas the chemical potential gradient causing the diffusion of K*G* from *A* to *C* will depend upon the value of N_{KG} in *C* the potential or osmotic pressure of the water in this phase will depend upon the total concentration of solute in *C*. Since the chief solute in *C* is $KHCO_3$ the diffusion of K*G* from *A* to *C* with subsequent conversion into $KHCO_3$ will eventually lower the activity of the water in *C* to a value below that in *A*. The effect of this upon the diffusion of water will now be considered.

At the beginning of the experiment, when the concentration of solute in *C* is lower than in *A*, water will diffuse from *C* into *A*. This initial movement of H_2O from C to A will continue until the diffusion of KG in the reverse direction, followed by its conversion into $KHCO_3$ in C, builds up the solute concentration in this phase to such a value that the activities of the water in A and C are equal. The activity of KG in C, however, which corresponds to this concentration of $KHCO_3$ is still much lower than the activity of KG in A so that KG continues to enter C and be converted into $KHCO_3$. This lowers the activity of H_2O in C below its value in A and the water consequently reverses its direction of flow and henceforth moves from A to C. The time at which this reversal occurs corresponds to a minimum in the water content of the phase C and is treated further in Section IX.

The simultaneous movement of KG and H_2O from A to C then continues indefinitely and approaches a condition in which KG and H_2O enter C in essentially the same ratio as that of $KHCO_3$ and H_2O already present, so that on conversion of the entering KG into $KHCO_3$ the concentration of this latter substance in C remains unaltered. This condition is called the steady state and is an important feature of the experiment.

Since it is impossible to fix rigidly the phase B in the model, the hydrodynamic pressure developed by the increase in the volume of C is exactly compensated by a shift in the position of the intermediate phase and must consequently be neglected in the present theory.

III

The Concentration Scale

As will be shown later, it is necessary to consider the partition of the diffusing constituents between the various phases and since one of these, water, is that which, in the outer aqueous layers, would normally be termed the solvent a concentration scale must be selected which is symmetrical with respect to all constituents. The mole fraction satisfies this requirement and will therefore be adopted. As indicated in equation (2.2) N_i represents the mole fraction of the i-th constituent while the actual number of moles of i present in any given region will be denoted by n_i. The mole fraction is defined by the expression

$$N_i = \frac{n_i}{n_0 + n_1 + n_2 + \cdots} = \frac{n_i}{\Sigma n_i} \qquad (3.1)$$

in which the summation is to be taken over all species present. The mole fraction is related to the volume concentration (c_i moles per milliliter of solution) by the equation

$$c_i = \frac{n_i}{\Sigma\, n_i\, \overline{V}_i}$$

in which $\overline{V}_i$ is the partial molal volume of the i-th constituent in milliliters.

If one constituent of a phase is greatly predominant it may be represented by the subscript 0 and no serious error is involved in replacing the mole fraction of another constituent by its mole ratio, $\frac{n_i}{n_0}$,

In this case

$$\left.\begin{aligned} N_i &\simeq \frac{n_i}{n_0};\ N_0 \simeq 1 - \frac{\Sigma\, n_i}{n_0} && (3.2)\\ c_i &\simeq \frac{n_i}{n_0 V_0} \simeq \frac{N_i}{V_0} && (3.3) \end{aligned}\right\} i \neq 0$$

in which V_0 is the molal volume of the "solvent."

IV

The Diffusion Equation

In the derivation of the equations which describe molecular diffusion processes it is customary to assume that the velocity of migration, v_i, of the i-th constituent is proportional to the chemical potential gradient, $\frac{d\mu_i}{dx}$, of that substance[6]

$$v_i = -\, u_i \frac{d\mu_i}{dx} \tag{4.1}$$

u_i being the factor of proportionality or mobility. The derivatives of μ_i with respect to the other space coordinates may be neglected if diffusion takes place in one direction only as in the present experiment. Equation (4.1) may be multiplied by N_i and rearranged to

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[6] Onsager, L., and Fuoss, R. M., *J. Phys. Chem.*, 1932, **36**, 2689 (see page 2759).

$$v_iN_i = - u_iN_i \frac{d\mu_i}{dN_i} \frac{dN_i}{dx} \tag{4.2}$$

Since this equation is to be applied only to the substances diffusing through B and since these are present in this phase at relatively low volume concentrations the approximation represented by equation (3.3) may be introduced into equation (4.2) to give

$$v_ic_i = - \frac{u_iN_i}{V_0} \frac{d\mu_i}{dN_i} \frac{dN_i}{dx}$$

It is evident, however, that the product v_ic_i is the velocity with which a given concentration moves and hence is the flux of material per unit area and unit time, $\frac{\partial n_i}{\partial t}$, through a plane perpendicular to the direction of migration. If A is the total cross-section of the diffusion layer the diffusion equation becomes

$$\frac{\partial n_i}{\partial t} = - \frac{A}{V_0} \left(u_iN_i \frac{d\mu_i}{dN_i} \right) \frac{\partial N_i}{\partial x} \tag{4.3}$$

Equation (4.3) will be recognized as Fick's law if

$$D_i = u_iN_i \frac{d\mu_i}{dN_i}$$

D_i being the diffusion coefficient. By means of equation (2.2) this may be rearranged to

$$D_i = RT\, u_i \left(1 + \frac{d \ln \gamma_i}{d \ln N_i} \right) \tag{4.4}$$

Since $\frac{d \ln \gamma_i}{d \ln N_i}$ is generally a function of N_i it is evident that the diffusion coefficient will also vary with the concentration. However, it has already been assumed that diffusion occurs only in the non-aqueous phase, in which, due to the low ionizing power of the solvent, electrolytes behave more nearly as perfect solutes than in an aqueous phase. For a perfect solute the differential coefficient in equation (4.4) is zero and in the present instance may at least be assumed constant.

Moreover, it simplifies the mathematical treatment if the very probable assumption is made that at any given time the thickness of the combined diffusion layers (*aa′* and *bb′* of Fig. 1) has a value sufficiently small to warrant the consideration of the concentration gradient as constant and equal to $\frac{\Delta N_i}{\Delta x}$. Since the value of Δx is nearly independent of the time, equation (4.3) may be rewritten

$$\frac{dn_i}{dt} = -\frac{AD_i}{V_0 \cdot \Delta x}\Delta N_i \tag{4.5}$$

V

Equilibria at the Phase Boundaries

Equation (4.5) is the general differential equation which will be adapted to the experiment considered in this paper. Before this adaptation can be made, however, the mechanism of the transfer of material across the phase boundaries must be considered. If, as is usually assumed, equilibrium for a given constituent is attained practically instantaneously at a phase boundary, it is correct to say that the chemical potential for that substance is equal in the two phases at the boundary and hence has no discontinuity at that place.

According to equation (4.1) the velocity of a constituent is proportional to the gradient of chemical potential, the latter being continuous at a phase boundary as indicated above, but the flux of a constituent is determined not only by its velocity but by the product of this into the concentration which is usually discontinuous at a phase boundary. It thus appears that the driving force which causes diffusion across the non-aqueous layer will be determined largely by the composition of the two aqueous phases, whereas the flux of material will depend in part upon the concentration of the diffusing substances in the non-aqueous layer. Thus, other conditions being the same, a given difference of composition between *A* and *C* will cause a greater flux of some constituent through *B* the more soluble that constituent is in the non-aqueous layer. As Osterhout[2] has emphasized, partition coefficients will therefore play an important rôle in the present theory and may be defined for the phase boundaries *a* and *b* by the relations

$$S_i^a = N_i^{Ba}/N_i^A \tag{5.1}$$

$$S_i^b = N_i^{Bb}/N_i^C \tag{5.2}$$

In these expressions the superscript denotes the region to which the concentration or partition coefficient refers. Thus N_i^{Ba} is the concentration of the i-th constituent in phase B at the plane a. These are stoichiometric partition coefficients and are functions of the concentration in general.

Since the aqueous layer A is continuously replaced, N_{KG}^A, $N_{H_2O}^A$ and the corresponding partition coefficients, S_{KG}^a and $S_{H_2O}^a$, are constants, the values of the latter being 1.7[4] and 0.417[7] respectively. At the phase boundary b, however, the concentrations, and hence the partition coefficients, vary with the time and this variation on the part of the partition coefficients must now be discussed in some detail.

In the absence of data on the effect of salts upon the distribution of the non-electrolyte, water, the coefficient $S_{H_2O}^b$ may be considered constant—and equal to 0.417, the value obtained from the mutual solubility of the two liquids, HG and H_2O, in the absence of salts—if the effect of the salt ionization upon the activity of water in the aqueous phase is considered. This may be done by introducing the osmotic coefficient, i, into the expressions for $N_{H_2O}^A$ and $N_{H_2O}^C$, as follows:

$$N_{H_2O}^C \simeq 1 - N_{HG}^C - N_{CO_2}^C - i^C N_{KHCO_3}^C \tag{5.3}$$

$$N_{H_2O}^A \simeq 1 - N_{HG}^A - i^A N_{KG}^A \tag{5.4}$$

The osmotic coefficient is also a function of the concentration but no serious error will be made if an average but constant value is assigned to it for the particular experiment under consideration. Thus a value of 1.9 may be assigned to i^A, corresponding to the constant value of $N_{KG}^A = 0.00090$, and $i^C \simeq 1.7$ which corresponds to the average value $N_{KHCO_3}^C = 0.006$ (see Table I). The values of N_{HG}^A ($= N_{HG}^C$) and $N_{CO_2}^C$ are 0.0027[1] and 0.00062,[8] respectively.

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[7] Unpublished measurements made in this laboratory. The solubility of water in the non-aqueous material is about 9.73 per cent. Thus 100 gm. of the non-aqueous phase contain 9.73 gm. of H_2O or 0.54 mole, and 90.3 gm. of HG or 0.756 mole (see Footnote 12). $N_{H_2O}^{Ba}$ is therefore $\frac{0.54}{0.54 + 0.756}$ or 0.417 and since $N_{H_2O}^A$ is essentially unity, $S_{H_2O}^a = 0.417$.

[8] International Critical Tables, New York, McGraw-Hill Book Co., Inc., 1928, **3**, 260.

Osterhout, Kamerling, and Stanley[4] have found that the distribution of KG between HG and H_2O is quite sensitive to the concentration when this is low but becomes nearly independent of the latter as saturation is approached. The exact functional relationship has not been established, however, and in the present theory a constant value of 1.85 will be assigned to S^b_{KG}. The use of a constant value for S^b_{KG} in this manner is clearly unsatisfactory and probably represents a rather poor approximation, but the mathematical complexity which results from the assumption of even a linear variation of the partition coefficient with concentration is rather serious. Moreover the common ion effect of the potassium ion from the $KHCO_3$ in C upon the partition of KG introduces an additional complication. The value which has been assigned to S^b_{KG} was obtained in the following manner.

According to the conventions of Lewis and Randall[5] the activity of KG in the phase C is given by the relation

$$a^C_{KG} = a^C_{K^+} \cdot a^C_{G^-} = (\gamma^C_\pm)^2 \, N^C_{K^+} \cdot N^C_{G^-}$$

in which $\gamma^C_\pm$ is the mean ion activity coefficient and will depend upon the total ionic strength of phase C. The distribution measurements of Osterhout, Kamerling, and Stanley were made with solutions of pure KG and in this case,

$$\bar{N}^C_{K^+} = \bar{N}^C_{G^-} = \bar{N}^C_{KG}$$

and

$$a^C_{KG} = (\bar{\gamma}^C_\pm)^2 \, (\bar{N}^C_{KG})^2 \tag{5.5}$$

the bar over the symbol indicating that KG is the only salt present. In the experiment being considered in this paper, however, $KHCO_3$ is also present in C at an average concentration of $N^C_{KHCO_3} = 0.006$, so that

$$N^C_{K^+} = N^C_{KHCO_3} + N^C_{KG}$$
$$N^C_{G^-} = N^C_{KG}$$

and

$$a^C_{KG} = (\gamma^C_\pm)^2 \, N^C_{KG} \, (N^C_{KHCO_3} + N^C_{KG}) \tag{5.6}$$

Elimination of a^C_{KG} between equations (5.5) and (5.6) gives

$$\overline{N}^C_{KG} = \frac{\gamma^C_\pm}{\overline{\gamma}^C_\pm}\sqrt{N^C_{KG}\,(N^C_{KHCO_3} + N^C_{KG})} \tag{5.7}$$

or with reference to equation (2.6)

$$\overline{N}^C_{KG} = \frac{\gamma^C_\pm}{\overline{\gamma}^C_\pm}\,N^C_{KHCO_3}\sqrt{\frac{1}{K} + \frac{1}{K^2}}$$

By means of equations (6.8) and (8.4) which follow, K may be eliminated to give

$$\overline{N}^C_{KG} = \frac{\gamma^C_\pm}{\overline{\gamma}^C_\pm}\,N^C_{KHCO_3}\sqrt{\frac{0.0876}{S^b_{KG}} + \frac{0.00768}{(S^b_{KG})^2}}$$

The value of S^b_{KG} corresponding to this value of $\overline{N}^C_{KG}$, as obtained from a plot of the partition measurements mentioned above, is the quantity desired. Since S^b_{KG} must be known, however, before $\overline{N}^C_{KG}$ can be evaluated and since $\overline{\gamma}^C_\pm$ also depends upon N^C_{KG}, a series of approximations will be necessary. For $N^C_{KHCO_3} = 0.006$, $\gamma^C_\pm \sim 0.59$.[9] The computed value $\overline{N}^C_{KG}$ is 0.00106, so that $S^b_{KG} = 1.85$ and $\overline{\gamma}^C_\pm \sim 0.73$ if the activity coefficients of K*G* are similar to those for $KHCO_3$ and if the small amount of H*G* present in the aqueous phase *C* may be ignored. In the steady state $N^C_{KHCO_3} = 0.012$ and a similar calculation gives the value 2.5 for S^b_{KG}. The difference between 1.85 and 2.5 affords an estimate of the error which is involved in the assignment of a constant value to S^b_{KG}.

It has been necessary to develop the foregoing theory in order to utilize the results of ordinary partition measurements in obtaining the distribution equilibria in the presence of an added salt with a common ion. It should be apparent that the theory is also applicable when a salt with no ion in common is added to the aqueous phase. The effect upon the partition equilibria is then contained entirely in the activity coefficient ratio of equation (5.7). However, it should be emphasized that the added salt cannot be appreciably soluble in the non-aqueous phase if the foregoing equations are to be applicable.

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[9] Guntelberg, E., and Schiödt, E., *Z. phys. Chem.*, 1928, **135**, 393.

VI

Specialization of the Diffusion Equation

The flux of the two diffusing substances through the plane b, Fig. 1, will now be considered. The flux of KG through this plane is, according to equation (4.5),

$$\left(\frac{dn_{\mathrm{K}G}}{dt}\right)_b = \frac{AD_{\mathrm{K}G}}{V_{\mathrm{H}G}\Delta x}\left(N^{Ba}_{\mathrm{K}G} - N^{Bb}_{\mathrm{K}G}\right)$$

From equations (5.1) and (5.2), this may also be written

$$\left(\frac{dn_{\mathrm{K}G}}{dt}\right)_b = \frac{AD_{\mathrm{K}G}}{V_{\mathrm{H}G}\Delta x}\left(S^a_{\mathrm{K}G}\, N^A_{\mathrm{K}G} - S^b_{\mathrm{K}G}\, N^C_{\mathrm{K}G}\right) \tag{6.1}$$

Due to the reaction (see equation (2.3)) which occurs in C the flux of KG through the plane b is essentially the rate of appearance of $KHCO_3$ in C

$$\left(\frac{dn_{\mathrm{K}G}}{dt}\right)_b = \frac{dn^C_{\mathrm{KHCO_3}}}{dt}$$

Elimination of $N^C_{\mathrm{K}G}$ between equations (6.1) and (2.6) gives

$$\frac{dn^C_{\mathrm{KHCO_3}}}{dt} = \frac{AD_{\mathrm{K}G}}{V_{\mathrm{H}G}\Delta x}\left(S^a_{\mathrm{K}G}\, N^A_{\mathrm{K}G} - \frac{S^b_{\mathrm{K}G}}{K}\, N^C_{\mathrm{KHCO_3}}\right) \tag{6.2}$$

Similarly the rate of appearance of water in C is

$$\frac{dn^C_{\mathrm{H_2O}}}{dt} = \frac{AD_{\mathrm{H_2O}}}{V_{\mathrm{H}G}\Delta x}\left(S^a_{\mathrm{H_2O}}\, N^A_{\mathrm{H_2O}} - S^b_{\mathrm{H_2O}}\, N^C_{\mathrm{H_2O}}\right) \tag{6.3}$$

Since the partition coefficients are to be considered constant, reference to equations (3.2), (5.3), and (5.4) shows that the number of dependent variables in equations (6.2) and (6.3) may be reduced to two as follows:

$$\frac{dn^C_{\mathrm{KHCO_3}}}{dt} = \frac{AD_{\mathrm{K}G}}{V_{\mathrm{H}G}\Delta x}\left(S^a_{\mathrm{K}G}\, N^A_{\mathrm{K}G} - \frac{S^b_{\mathrm{K}G}}{K}\,\frac{n^C_{\mathrm{KHCO_3}}}{n^C_{\mathrm{H_2O}}}\right)$$

$$\frac{dn^C_{\mathrm{H_2O}}}{dt} = \frac{AD_{\mathrm{H_2O}}}{V_{\mathrm{H}G}\Delta x}\, S_{\mathrm{H_2O}}\left[\left(1 - N^A_{\mathrm{H}G} - i^A N^A_{\mathrm{K}G}\right) - \left(1 - N^C_{\mathrm{H}G} - N^C_{\mathrm{CO_2}} - i^C\,\frac{n^C_{\mathrm{KHCO_3}}}{n^C_{\mathrm{H_2O}}}\right)\right]$$

$$= \frac{AD_{\mathrm{H_2O}}}{V_{\mathrm{H}G}\Delta x}\, S_{\mathrm{H_2O}}\left(-\, i^A\, N^A_{\mathrm{K}G} + N^C_{\mathrm{CO_2}} + i^C\,\frac{n^C_{\mathrm{KHCO_3}}}{n^C_{\mathrm{H_2O}}}\right)$$

The notation may be simplified by placing

$$n^C_{\mathrm{KHCO_3}} = n_1 \tag{6.4}$$

$$n^C_{\mathrm{H_2O}} = n_0 \tag{6.5}$$

and by collecting the various constants as follows:

$$\frac{AD_{\mathrm{KG}}}{V_{\mathrm{HG}}\Delta x}\,\frac{S^b_{\mathrm{KG}}}{K} = \alpha_1 \tag{6.6}$$

$$\frac{AD_{\mathrm{H_2O}}}{V_{\mathrm{HG}}\Delta x}\,S_{\mathrm{H_2O}} \cdot i^C = \alpha_0 \tag{6.7}$$

$$N^A_{\mathrm{KG}}\,K \cdot S^a_{\mathrm{KG}}/S^b_{\mathrm{KG}} = \beta_1 \tag{6.8}$$

$$N^C_{\mathrm{CO_2}}/i^C - N^A_{\mathrm{KG}}\,i^A/i^C = -\beta_0 \tag{6.9}$$

Then

$$\frac{dn_1}{dt} = \alpha_1\left(\beta_1 - \frac{n_1}{n_0}\right) \tag{6.10}$$

$$\frac{dn_0}{dt} = \alpha_0\left(-\beta_0 + \frac{n_1}{n_0}\right) \tag{6.11}$$

VII

Solution of the Differential Equations

Elimination of n_1/n_0 between equations (6.10) and (6.11) gives the expression

$$\frac{1}{\alpha_1}\frac{dn_1}{dt} + \frac{1}{\alpha_0}\frac{dn_0}{dt} = \beta_1 - \beta_0$$

which may be immediately integrated to give

$$\frac{n_1}{\alpha_1} + \frac{n_0}{\alpha_0} = (\beta_1 - \beta_0)t + \frac{I_0}{\alpha_0} \tag{7.1}$$

$\dfrac{I_0}{\alpha_0}$ is a constant of integration, I_0 being merely the number of moles of water initially present in phase C since the value of n_1 is zero at this time.

Translation of the time axis by means of the substitution

$$t = t' - \frac{I_0}{\alpha_0(\beta_1 - \beta_0)}$$

reduces equation (7.1) to

$$\frac{n_1}{\alpha_1} + \frac{n_0}{\alpha_0} = (\beta_1 - \beta_0)t' \tag{7.2}$$

Equation (7.2) tells us nothing about the individual dependence of n_1 and n_0 upon the time, but by solving this expression for n_1 and substituting this value into equation (6.11) an expression involving only one dependent variable is obtained. Thus,

$$n_1 = \alpha_1(\beta_1 - \beta_0)t' - \frac{\alpha_1}{\alpha_0} n_0$$

and

$$\frac{dn_0}{dt'} = -\alpha_0\beta_0 - \alpha_1 + \alpha_1\alpha_0(\beta_1 - \beta_0)\frac{t'}{n_0} \tag{7.3}$$

Equation (7.3) is homogeneous and the variables are rendered separable by the substitution

$$n_0 = v \cdot t' \tag{7.4}$$

so that it becomes

$$vdt' + t'dv = -bdt' - a\frac{dt'}{v}$$

if

$$-a = \alpha_1\alpha_0(\beta_1 - \beta_0) \tag{7.5}$$

$$b = \alpha_1 + \alpha_0\beta_0 \tag{7.6}$$

Rationalization and separation of the variables gives the expression

$$\frac{vdv}{a + bv + v^2} + \frac{dt'}{t'} = 0$$

The integral of this is

$$\ln(a + bv + v^2) - \frac{b}{\sqrt{-q}}\ln\frac{2v + b - \sqrt{-q}}{2v + b + \sqrt{-q}} + 2\ln I_1' t' = 0 \tag{7.7}$$

in which I_1' is a constant of integration and $q = 4a - b^2$. Since

$$4(a + bv + v^2) = (2v + b - \sqrt{-q})(2v + b + \sqrt{-q})$$

equation (7.7) may be rearranged to

$$\left(1 - \frac{b}{\sqrt{-q}}\right) \log (2v + b - \sqrt{-q}) + \left(1 + \frac{b}{\sqrt{-q}}\right) \log (2v + b + \sqrt{-q}) + 2 \log t' + I_1 = 0 \qquad (7.8)$$

In equation (7.8) Briggsian logarithms have been substituted throughout for the natural logarithms and it is in this form that computations can be made most conveniently. This equation cannot be solved explicitly for v, and hence for n_0, but it is already explicit in t' and therefore may be readily employed for a calculation of the time curves as follows:

From equations (7.2) and (7.4),

$$N_1 \simeq \frac{n_1}{n_0} = \alpha_1(\beta_1 - \beta_0) \frac{t'}{n_0} - \frac{\alpha_1}{\alpha_0} = \alpha_1(\beta_1 - \beta_0) \frac{1}{v} - \frac{\alpha_1}{\alpha_0} \qquad (7.9)$$

and a value of v may be computed which corresponds to a given concentration, N_1. This value of v is substituted in equation (7.8) and the corresponding value of t' computed. This value of t', together with the value of v which was taken, is then substituted in equation (7.4) and n_0 computed, etc.

Before these computations can actually be made, however, values must be assigned to the necessary constants. The constant I_0, as shown above, is the number of moles of water initially present in phase C and has the value 3.321. A value of 0.00064 for β_0 may be computed from equation (6.9) since all of the terms on the left hand side of this expression are known. After values have been assigned to the other necessary constants the integration constant I_1 may be evaluated by means of equation (7.8) from the conditions that at zero time $t' = \dfrac{I_0}{\alpha_0(\beta_1 - \beta_0)}$ and $v = \dfrac{I_0}{t'}$ (equation (7.4)). The value of I_1 which was used in the computations for the time curves is -2.202297. The constants α_0, α_1, and β_1 involve the characteristic but unknown quantities D_{KG}, D_{H_2O}, Δx, and K, and must therefore be evaluated from the experimental data. A method of evaluating these constants will be outlined in the next section.

VIII

Evaluation of the Characteristic Constants

The data recorded by Osterhout were the volumes, V, in milliliters, of the aqueous phase C and the total salt normality in this phase for different values of the time, t, in hours. These data are recorded in the first three columns of Table I. Values of n_1, and n_0, which are recorded in Columns 5 and 6 of the table, were computed by means of the relations

$$n_1 = C \cdot V$$

$$n_0 = \frac{V \cdot d - 100.1\, n_1}{18.015}$$

100.1 and 18.015 being the molar weights of $KHCO_3$ and H_2O respectively. d is the density of the solution and was taken as equal to that of a pure aqueous solution of $KHCO_3$ as given in the International Critical Tables.[10] The values of the density are recorded in the fourth column.

The data of Table I may be used to evaluate the constants α_1, α_0, and β_1 as follows: Since equation (7.1) may be rearranged to

$$\frac{n_0 - I_0}{n_1} = \frac{n_0 - 3.321}{n_1} = \alpha_0(\beta_1 - \beta_0)\frac{t}{n_1} - \frac{\alpha_0}{\alpha_1}$$

a plot of $\frac{n_0 - 3.321}{n_1}$ as ordinate against $\frac{t}{n_1}$ as abscissae should give a straight line with a slope, $\alpha_0(\beta_1 - 0.00064)$, and an intercept, $-\alpha_0/\alpha_1$. This method of plotting the data (Fig. 2) magnifies small experimental errors in the initial stages of the experiment when n_0 differs but little from 3.321 and it is therefore not surprising that the first two points in Fig. 2 deviate considerably from a straight line. The remaining points yield a satisfactory straight line although such a line is not drawn in the figure. Since this plot is to be used for the evaluation of two of the constants which occur in the theory the final time curves for the entrance of salt and water can best be represented if all points in Fig. 2 are given equal weight. When this is done the straight line which best represents the data, as determined by the method of least squares, is the line drawn in the figure. The slope and intercept of this line furnish the relations

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[10] International Critical Tables, New York, McGraw-Hill Book Co., Inc., 1928, **3**, 90.

TABLE I

Experimental Data

1	2	3	4	5	6	7
t	KHCO$_3$ conc.	V	d^{25}_4 density	$n_1 = n^C_{\mathrm{KHCO_3}}$	$n_0 = n^C_{\mathrm{H_2O}}$	$\frac{n_1}{n_0} \approx N_1 = N^C_{\mathrm{KHCO_3}}$
hrs.	*mole/liter*	*ml.*				
0	0.00	60	0.997	0.0000	3.321	0.0000
16	0.10	60	1.004	0.0060	3.311	0.00181
42	0.26	64	1.014	0.01664	3.510	0.00474
65	0.40	68	1.023	0.0272	3.710	0.00733
80	0.46	72	1.027	0.03312	3.930	0.00843
104	0.53	76	1.032	0.04028	4.130	0.00975
128	0.60	81	1.037	0.0486	4.393	0.01106
151	0.61	87	1.037	0.05307	4.713	0.01126
178	0.62	93	1.038	0.05766	5.038	0.01145
208	0.63	99	1.038	0.06237	5.358	0.01164
232	0.63	105	1.038	0.06615	5.685	0.01164
256	0.63	112	1.038	0.07056	6.062	0.01164

$$\alpha_0/\alpha_1 = 38.65 \tag{8.1}$$

$$\alpha_0(\beta_1 - 0.00064) = 0.02128 \tag{8.2}$$

A third relation between the three constants is necessary and may be obtained from the limiting slope of the n_0-t curve as the steady state is approached. The straight line of Fig. 3 appeared to have the correct slope, the value being

$$\left(\frac{dn_0}{dt}\right)_{\frac{n_1}{n_0} = 0.01164} = 0.01392 = \alpha_0(0.01164 - 0.00064) \tag{8.3}$$

Equations (8.1), (8.2), and (8.3) then give

$$\alpha_0 = 1.265$$
$$\alpha_1 = 0.03273 \tag{8.4}$$
$$\beta_1 = 0.01746$$

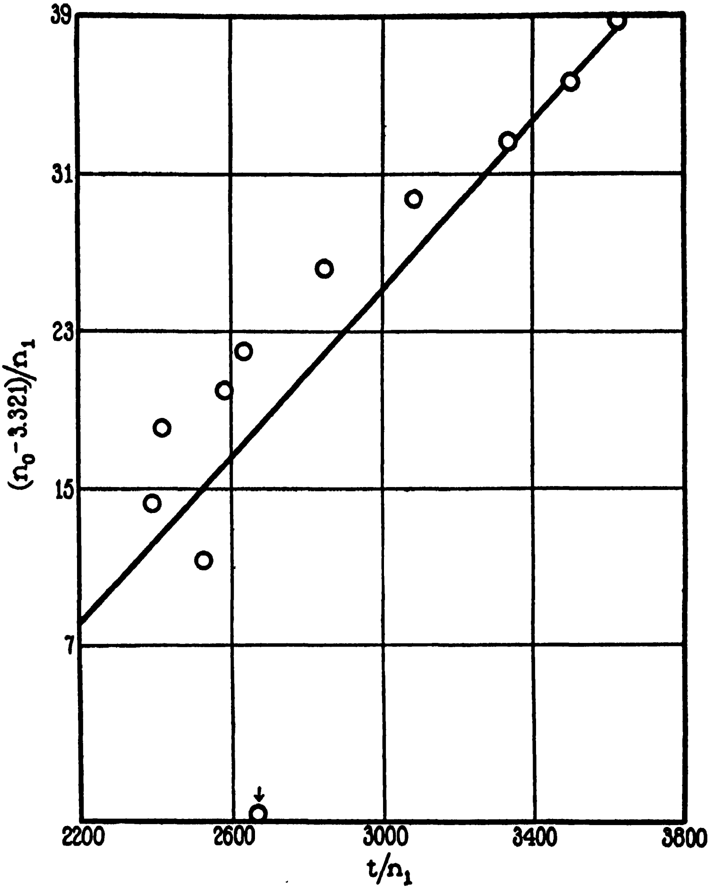

FIG. 2. A rectilinear plot of certain functions of the experimental data. From the slope and intercept of this line two of the three empirical constants which occur in the theory may be evaluated.

With these values of the constants the time curves may be computed by the method which has been outlined. The results of these computations are given in Table II and are plotted as smooth curves in Figs. 3 and 4.

TABLE II

Computed Values of n_0, n_1, and t

N_1	t	n_1	n_0
0.00064	3.77	0.0021	3.319_5
0.002	12.34	0.0066	3.327
0.004	26.80	0.0135	3.371
0.006	44.87	0.0208	3.471
0.008	70.09	0.0294	3.676
0.010	114.45	0.0415	4.152
0.0110	161.70	0.0522	4.745
0.0115	211.43	0.0623	5.414
0.01175	261.06	0.0717	6.104
0.0120	399.70	0.0970	8.079

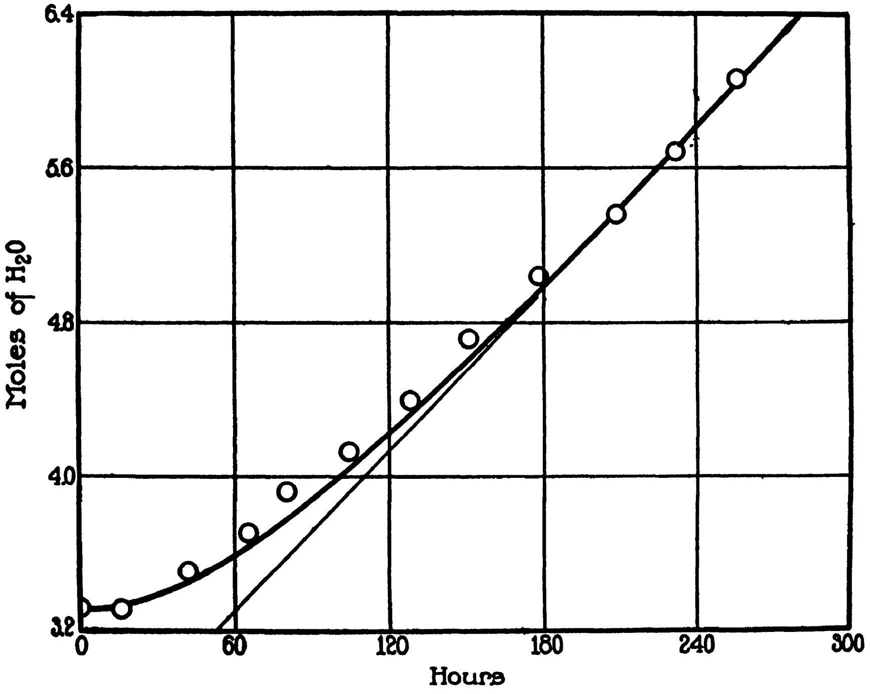

FIG. 3. A comparison of theory with experiment for the change of the water content of phase C with the time. The theoretical curve as computed from equation (7.8) is drawn as a full heavy line and the experimental points are indicated by the circles. The asymptote to this curve as the steady state is approached is drawn as a straight light line and the slope of this line was used in the evaluation of one of the constants of the theory.

IX

Comparison of Theory with Experiment

The computed time curve for the entrance of water is in fair agreement with the experimental data which are plotted as circles in Fig. 3. Thus the theory predicts the minimum in this curve after the experiment has been in progress for a few hours. It will be recalled that this minimum occurs at the time that the water reverses its initial direction of flow and begins to move from A to C (Fig. 1). At this time

$$\frac{dn_0}{dt} = \alpha_0\left(-\beta_0 + \frac{n_1}{n_0}\right) = 0$$

and

$$\left(\frac{n_1}{n_0}\right) = \beta_0 = 0.00064$$

The values of n_0 and t corresponding to this value for $\frac{n_1}{n_0}$ are 3.319 and 3.77 respectively. Phase C thus has a minimum water content after about 4 hours.

Agreement with experiment in the case of the concentration-time curve is satisfactory as may be seen by reference to Fig. 4. An essential feature of the experiment—the approach to the steady state—is clearly illustrated. The characteristics of the steady state may be demonstrated as follows: Differentiation of the relation

$$N_1 = \frac{n_1}{n_0}$$

with respect to the time yields the expression

$$n_0 \frac{dN_1}{dt} + N_1 \frac{dn_0}{dt} = \frac{dn_1}{dt} \tag{9.1}$$

In the steady state N_1 is constant and equal to N_{1s}. Moreover $\frac{dN_1}{dt} = 0$ so that equation (9.1) becomes

$$N_{1s}\left(\frac{dn_0}{dt}\right)_s = \left(\frac{dn_1}{dt}\right)_s$$

From equations (6.10) and (6.11), however,

$$N_{1s}\,\alpha_0(-\beta_0 + N_{1s}) = \alpha_1(\beta_1 - N_{1s})$$

Solution of this quadratic gives, as the only physically possible root, the expression

$$N_{1s} = \frac{1}{2\alpha_0}\left(\alpha_0\beta_0 - \alpha_1 + \sqrt{(\alpha_1 - \alpha_0\beta_0)^2 + 4\alpha_0\alpha_1\beta_1}\right) \qquad (9.2)$$

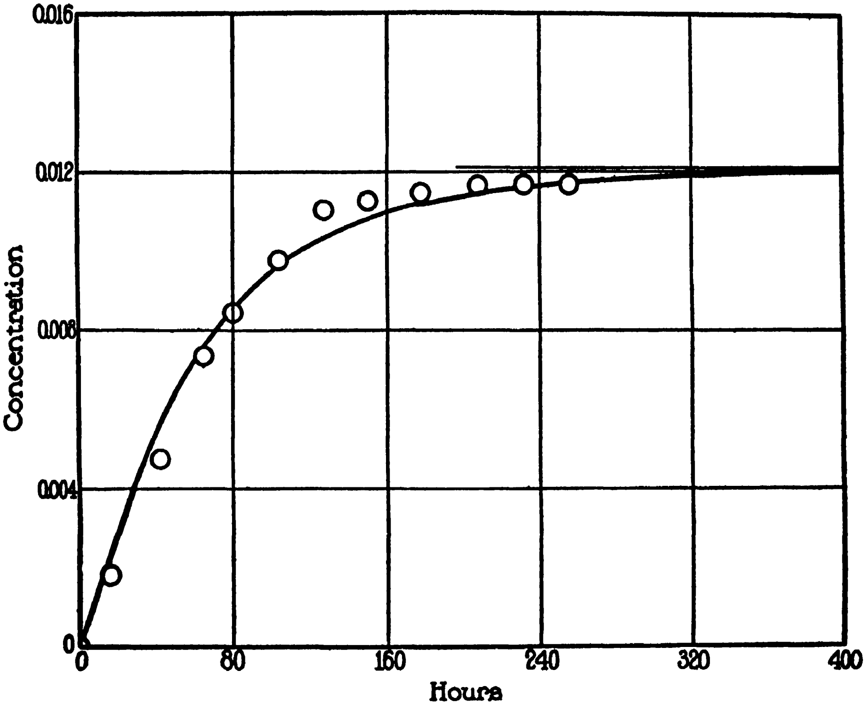

FIG. 4. A comparison of theory with experiment for the change of salt concentration in phase C with the time. The theoretical curve is drawn as a full heavy line and the experimental points are indicated by the circles. Concentrations are expressed as mole fractions and the light horizontal line is the theoretical value of the concentration in the steady state.

Substitution of numerical values for α_0, etc., leads to a value for N_{1s} of 0.0121 which is but slightly greater than the final values (0.011_6) which were observed during the latter stages of the experiment.

Through a combination of equations (9.2), (7.9), and (7.8) it may be shown that the steady state concentration is attained only after infinite time, but a comparison of the curve of Fig. 4 with this limiting asymptote which is drawn as a straight horizontal line indicates that the concentration has reached a value only 5 per cent less than the limiting value after about 200 hours.

X

CONCLUSION

Some of the physical quantities which appear in the expressions for α_0, α_1, and β_1 (equations (6.6), (6.7), and (6.8)) are capable of independent measurement and in conclusion a discussion of these constants will be given. Thus a value for K may be evaluated from certain properties of HG and H_2CO_3, namely, the solubilities of these weak acids in water and their ionization constants.

In equation (2.5) both numerator and denominator may be multiplied by a_{H^+} to give

$$K = \frac{a_{HCO_3^-} \cdot a_{H^+}}{a_{G^-} \cdot a_{H^+}} \tag{10.1}$$

but $a_{HCO_3^-} \cdot a_{H^+} = K_{H_2CO_3} \cdot a_{H_2CO_3}$ and $a_{G^-} \cdot a_{H^+} = K_{HG} \cdot a_{HG}$ so that equation (10.1) may be written

$$K = \frac{K_{H_2CO_3} \cdot a_{H_2CO_3}}{K_{HG} \cdot a_{HG}} \tag{10.2}$$

Since $K_{H_2CO_3}$ has the value 4.54[11] $\times$ 10^{-7} and K_{HG} an even smaller value, $a_{H_2CO_3}$ may be replaced by the solubility of CO_2 at 1 atmosphere (0.034 mole/liter) and a_{HG} by the solubility of HG (0.15 mole/liter).

It is impossible to make an accurate estimation of K by means of equation (10.2) due to the fact that the weak acid represented by the symbol HG was, in the experiment of Osterhout and Stanley, a mixture of two weak acids, guaiacol (70 per cent) and *p*-cresol (30 per cent),[12] and also to the fact that the ionization constants for these substances which are to be found in the literature are very discordant. Thus a hydrolysis method[13] gives

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[11] MacInnes, D. A., and Belcher, D., *J. Am. Chem. Soc.*, 1933, **55**, 2630.

[12] In all computations involving this material, such as conversion of volume concentrations to mole fractions, etc., a molar weight, density, and molal volume of 119, 1.11, and 107, respectively, have been employed. These values are the means of the values for the pure substances, account being taken of their proportion by weight.

$$K(\text{guaiacol}) = 1.17 \times 10^{-10}$$

$$K(p\text{-cresol}) = 6.7 \times 10^{-11}$$

whereas a conductance method[14] gives

$$K(p\text{-cresol}) = 1.1 \times 10^{-8}$$

If guaiacol and *p*-cresol are assumed to have essentially the same ionization constants, as the results of hydrolysis measurements would indicate, K in equation (10.2) has the value 9×10^2 if 1.1×10^{-10} is assumed for $K_{\mathrm{H}G}$ and the value 9 if $K_{\mathrm{H}G} = 1.1 \times 10^{-8}$. The value of 21 which may be computed from equation (6.8) and which best fits the diffusion data is intermediate between the two independently computed values. The results of this computation are inconclusive except in so far as the independent estimate of K indicates that most of the KG which enters C is converted into $KHCO_3$.

From equations (6.6) and (6.7) it is evident that

$$\frac{AD_{\mathrm{K}G}}{V_{\mathrm{H}G}\Delta x} = \frac{\alpha_1 K}{S^b_{\mathrm{K}G}} = 0.37$$

$$\frac{AD_{\mathrm{H_2O}}}{V_{\mathrm{H}G}\Delta x} = \frac{\alpha_0}{S_{\mathrm{H_2O}} \cdot i^C} = 1.8$$

The ratio of the two diffusion coefficients is $D_{\mathrm{H_2O}}/D_{\mathrm{K}G} = 4.8$, a value which indicates that H_2O diffuses through the non-aqueous layer much more rapidly than KG. This ratio is somewhat higher than would be expected from a consideration of molecular size and may be due to the fact that the concentration of water in the non-aqueous phase is so high that certain assumptions which were made in the derivation of the diffusion equations are partially invalidated.

Osterhout, Kamerling,[4] and Stanley have shown that the diffusion of KG in H_2O is about twelve times as rapid as in HG. The value of D_{KG} for diffusion in water is not known but it will certainly be of the same order of magnitude as the diffusion coefficient for a potassium salt with a large organic anion such as potassium acetate, namely, 1×10^{-5} $\overline{\text{cm}}.^2$/second. Thus D_{KG} for diffusion in HG is of the order 1×10^{-6} $\overline{\text{cm}}.^2$/second or 3.6×10^{-3} $\overline{\text{cm}}.^2$/hour. The mean area of the two phase boundaries a and b (Fig. 1) was about 95 $\overline{\text{cm}}.^2$. Consequently

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[13] Landolt, H., and Börnstein, R., Physikalisch-chemische Tabellen, Berlin, Julius Springer, 5th edition, (Roth, W. A., and Scheel, K.), 1931, suppl. vol. **2**, pt. 2, pp. 1087–88.

[14] International Critical Tables, New York, McGraw-Hill Book Co., Inc., 1929, **6**, 281.

$$\Delta x = \frac{AD_{KG}}{0.37\ V_{HG}} = \frac{95 \times 3.6 \times 10^{-3}}{0.37 \times 107} = 0.01 \text{ cm.}$$

From direct microscopic observation Davis and Crandall[15] have estimated that the thickness of the unstirred water layer at a gas-water interface is about 0.04 cm. While the two values are by no means comparable it appears that the value for Δx which has just been computed is a physically possible one. It is worthy of note that this value is sufficiently low to justify the assumption of a linear concentration gradient in the unstirred layers which was made.

The theory which has been developed in this paper is subject to many obvious refinements and it may be necessary to amend certain aspects of the physical interpretation of the experiment. Thus there are many[16] who consider reaction velocities in heterogeneous systems to be determined by a slow attainment of equilibria at the phase boundaries and not by the time element in the diffusion across unstirred layers. It is important to note, however, that either picture will lead to essentially the same differential equations as those developed in this paper though, of course, the physical interpretation of the constants will differ.

XI

SUMMARY

The differential equations which describe the simultaneous diffusion of water and a salt in a cell model have been formulated and solved. The equations have been derived from the general laws which describe diffusion processes, thereby furnishing a physical interpretation for the constants which enter into the theory. The theoretical time curves for the two diffusing substances are in good agreement with the experimentally determined curves and accurately reproduce all of the essential characteristics of the experiment.

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[15] Davis, H. S., and Crandall, G. S., *J. Am. Chem. Soc.*, 1930, **52**, 3757.

[16] See, for example, Roller, P. S., *J. Phys. Chem.*, 1932, **36**, 1202.

INTERMITTENT STIMULATION BY LIGHT

II. THE MEASUREMENT OF CRITICAL FUSION FREQUENCY FOR THE HUMAN EYE

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I

Critical Frequency

A field which is illuminated intermittently at a sufficiently high frequency produces a visual sensation similar to that of a field which is illuminated continuously. The frequency of interruptions at which this fusion of visual impressions takes place is called the critical frequency of flicker. Under controlled conditions the determination of this critical fusion frequency may be made with considerable accuracy.

A large body of work has been done in an effort to describe the precise value of the critical frequency under a variety of circumstances. These measurements will be described and evaluated in the next paper of this series. Here it is enough to state that the influence of the various factors on critical frequency is not wholly clear at present, especially in those aspects whose theoretical significance is most interesting. We have therefore undertaken an investigation of this problem, the results of which are to be presented in this group of papers. This paper is concerned with apparatus and methods, both of which it is necessary to describe in detail because of their significant bearing on the character of the measurements obtained previously and at present.

The apparatus was designed to present to the observer a small field of light periodically interrupted and surrounded by a much larger field continuously illuminated, but otherwise the same as the interrupted field. The various parts of the apparatus are then concerned with controlling and recording the position, intensity, spectral composition, and frequency of interruption of the visual field in their relation to the determination of the critical frequency of flicker by specific portions of the observer's eye.

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II

Optical System

The arrangement of the apparatus is shown diagrammatically in Fig. 1. The source of light is a concentrated filament, 500 watt, projection Mazda lamp, running on 110 volts and 4.2 amperes direct current furnished by storage cells. The lamp is placed in a rectangular lamp house which possesses a circular opening 30 mm. in diameter in each of two adjacent walls. The openings are covered with ground glass, and serve now as two secondary sources of illumination.

The light from one is deflected 90° in its path, by a totally reflecting prism, and focussed by a lens into the plane of a rotating, sectored wheel with four 45° sectors removed. The diverging light then passes through a hole in the silvering of a photometer cube, immediately after which it is focussed by a lens on to the exit pupil. Between this last lens and the exit pupil there are (*a*) places for filters, of which we used both neutral and monochromatic, (*b*) a neutral, balanced, Eastman Kodak gelatine wedge, and (*c*) a very thin slip of glass, tilted so as to reflect a red fixation point into the eye looking through the exit pupil.

The light from the other ground glass of the lamp house passes through an identical optical system and eventually impinges on the photometer cube, where it is reflected from the silvered diagonal face, through the lens, filters, wedge, and glass slip, on to the exit pupil. All light paths, prisms, sector wheel, etc., are enclosed in blackened tubing or in blackened housing to reduce stray light to a minimum.

The exit pupil is a circular opening 1.8 mm. in diameter, and constitutes the artificial pupil through which all the observations are made. An eye looking into the exit pupil sees the photometer cube through the wedge, balancer, filters, and lens, and sees it bounded by the circular edge of the lens. The visual field is thus a circular area 10° in diameter with a circular hole in it, 2° in diameter. The hole in the silvering on the diagonal of the cube is actually an ellipse, so made that in front view it appears circular. The larger circular field surrounding this opening is illuminated with continuous light; the smaller, central observation field is illuminated with intermittent light whose frequency depends on the rate at which the sector wheel rotates.

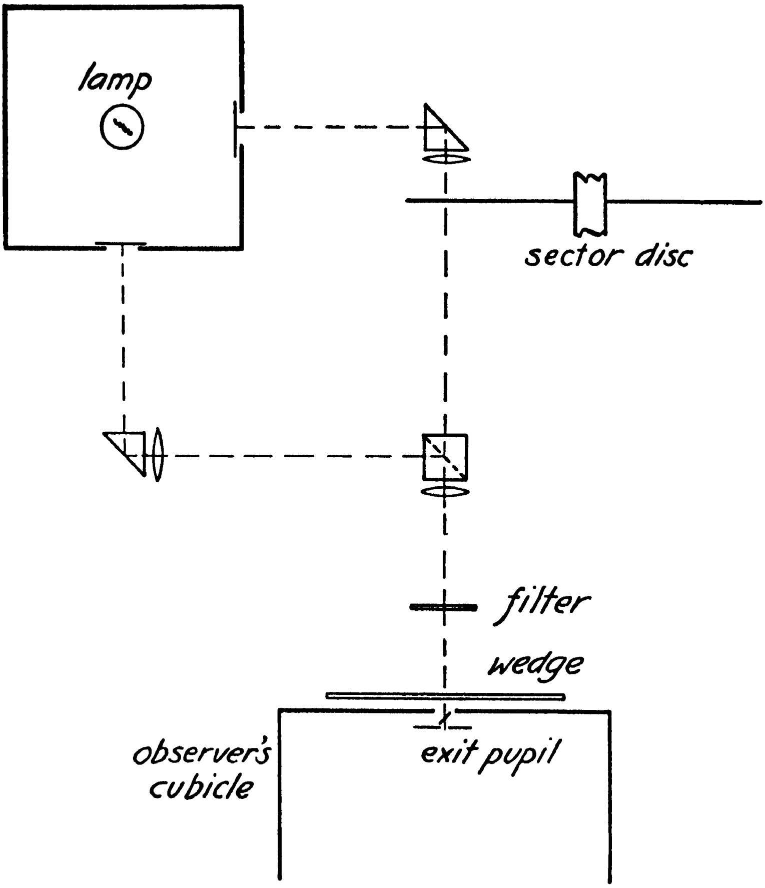

FIG. 1. A simplified, diagrammatic plan of the optical arrangements used in the measurements of critical frequency.

The filament of the Mazda lamp in the lamp house is in one plane. By rotating the lamp, one can vary the relative amount of light which falls on the two ground glass surfaces. This in turn controls the relative brightness of the inner, intermittently illuminated, test field, and the outer, continuously illuminated, surrounding field. In all our measurements we so adjusted the lamp that the brightness of the whole field was uniform; that is, that the brightness of the surrounding field was the same as the brightness of the test field when the sectored wheel was going well beyond the critical frequency of flicker, and the central field appeared continuously illuminated.

Our main reason for using the 10° surround of the same brightness as the experimental field is to be certain that the region surrounding the measuring area is in the same state of adaptation as the measuring area itself.[1] According to Lythgoe and Tansley (1929) the critical fusion frequency for most intensities becomes maximal when the surrounding field has the same brightness as the measuring field.

Fixation was provided for in several ways. In some of the measurements we used an ink spot on the lens in front of the cube. Later this was replaced by a luminous red fixation point reflected into the eye by a small cover-slip placed at an angle between the wedge and the exit pupil. The fixation point could then be adjusted for any desired visual angle by varying the tilt of the cover-slip. In the measurements made by C. D. V. on the field 20° off-center, a red fixation point was viewed through a prism by the *other*, non-measuring eye, and the binocular functioning of the two eyes was relied on to keep the working eye fixated.

The intensity of the light coming through the exit pupil is varied by means of two neutral, decimal, filters transmitting approximately

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[1] This point is frequently not appreciated, as, for example, in the recent work of Wilcox (1932). In this, the distance between two extremely small, illuminated stripes viewed *against an absolutely dark background* is used for measuring the relation of visual acuity to illumination. By this method Wilcox secures results which, at high illuminations of the stripes, do not resemble those obtained by previous workers; he therefore finds the theoretical treatment of such data inadequate.

Wilcox apparently does not realize that the term retinal illumination must bear some relation to the general light intensity prevailing on the retina in and around the area used for making the measurement. In Wilcox's case the total illuminated area of the two stripes occupies only 4.5 x 20 *minutes* of visual angle. Obviously one cannot know what the illumination on the retina is when, in the effort to resolve a small and very brightly illuminated test object, the eye moves here and there so that successive small retinal areas are now in very bright illumination and now in complete darkness. This is a problem in glare and not in visual acuity. Such a situation is here avoided by means of a comparatively large surround.

1/100 and 1/10,000 of the light, and a neutral wedge with a transmission range of 1 to 1000. The combination of wedge and neutral filters enables us to cover easily and accurately a range of illumination between 1 and 50,000,000 units. The spectral composition of the field is varied by placing Wratten Monochromatic Filters, Nos. 70 to 76, in the path of the light. The filters and wedge are from the Eastman Kodak Company.

The wedge is mounted so as to be moved by a rack and pinion, and is so arranged that its position may be read easily to 0.1 mm. on an attached millimeter scale. We calibrated the wedge by measuring the transmitted illumination at 13 points along its length by means of a Macbeth illuminometer. The relation between log I transmitted and distance along the wedge is linear, as is to be expected from the construction of the wedge.

The apparent brightness seen by the eye looking through the exit pupil, was measured by matching it against a white surface placed so as to cut off part of the visual field furnished by the lens. The illumination on the white surface was given by a lamp which was moved until the white surface, when viewed through the exit pupil, matched the rest of the field. The photometric brightness on the white surface was then measured directly with the illuminometer after the exit pupil had been removed. The factor for converting units of photometric brightness (millilamberts) into units of retinal illumination (photons) is $\pi a/10$ where a is the pupil area, and $\pi = 3.142$. With the present apparatus, which has a pupil area of 2.54 sq. mm. the maximum retinal illumination available in the central test area when it is not interrupted is very nearly 6000 photons.

The "neutral" filters we calibrated with a Martens polarization photometer for white light, and for each of the monochromatic filters. We first calibrated a 1/10 filter. Then keeping the 1/10 filter in place we put the 1/100 filter in the path of the other beam of the photometer. The difference between the two filters is then of the order of 1/10, which is readable with good accuracy on the scale of the polarization photometer. In the same way we calibrated a 1/1000 filter against the 1/100, and finally the 1/10,000 against the 1/1000 filter. In the measurements we used only the 1/100 and the 1/10,000 filters. The monochromatic filters we calibrated with a Koenig-Martens spectrophotometer.

III

Control of Frequency

The sector wheel, which is made of cold-rolled steel, is solidly mounted on an axle which rests in a special iron casting made to support and hold it. It can be actuated by either of two motors. For high speed its shaft is continuous with the shaft of a series-wound, high speed 1/8 horse power motor, while for low speeds it is driven by a 1/20 horse power, shunt-wound motor through a reducing gear and belt. The speed of the motors may be varied by means of rheostats in series with their power supply. The motors run on 110 volts, direct current supplied from storage cells.

There are two reasons for having two motors. One is that a single motor, unless it is run in connection with a variety of pulleys and wheels, cannot by variations in current supply be made to negotiate easily and smoothly the entire extent of frequencies which we desired. The other reason is that at low speeds we often wished to set a flicker frequency and to adjust the intensity of light until the flicker either just disappeared or just became visible. This requires the motor to remain quite steady, which can be accomplished by the shunt-wound motor. At higher frequencies we usually wished to set the intensity and to vary the speed of flicker. For this purpose it is desirable to have a motor which responds rapidly to change in power supply; this is accomplished by the series-wound motor.

It is of interest to determine the sharpness of cut-off and reappearance of the light by means of the sectored wheel. By clamping a long pointer perpendicularly to the long axis of the shaft of the sectored disc, we were able accurately to determine the angle through which the disc has to move in order for the visual field to pass from complete extinction to full intensity. This turns out to be nearly 3° of arc, and shows that the cut-off and reappearance of the illumination may be considered as practically rectangular.

For this work we wished to cover a rather large range of speeds for the rotation of the sector disc; in particular we wished to record the very slow speeds. We could find no commercially available tachometer which possessed both the desired range and the necessary accuracy. We therefore adopted the procedure of timing a given number of revolutions of the disc. In the beginning this was done by permanently connecting the shaft of the sectored disc with a revolution counter which made an audible contact every 100 revolutions through a relay system. The audible contacts were then timed with a stop-watch. This was for the faster speeds. For the slow speeds, the rotation of the shaft itself was directly observed, and 10 revolutions were timed with a stop-watch. Later the entire arrangement was made automatic by means of an electrical circuit a description of which follows.

IV

Automatic Timing

The system developed for automatically timing the rotational frequency of the sector disc consists essentially of three parts. The first is a small-angle contact on the shaft of the disc; this contact gives rise to one electrical impulse per revolution of the disc. The second part is an adding relay, through the primary circuit of which these impulses pass; the adding relay has a commutator on its face so arranged that its secondary circuit can be opened for a variable number of primary impulses. The third part is a Cenco impulse counter (Klopsteg, 1929) running on 60 cycle current; this impulse counter is used as a time-measuring device and is controlled through a polar relay by the secondary circuit of the adding relay. By this means the time occupied by a selected number of revolutions of the disc is automatically recorded in units of 1/120 of a second.

The range of flicker frequency covered in these experiments is from 2 to 60 per second. Since the sector disc gives 4 flicker cycles per revolution, the range of rotational frequency to be measured lies between $\frac{1}{4}$ and 15 revolutions per second. Since the inherent accuracy of the time-measuring system is 1/120 of a second, the shortest timing interval has to be about one second to achieve an accuracy of better than 1 per cent. This means that at the highest speeds of rotation 15 revolutions of the sector disc must be timed, whereas at the lowest speeds only 1 revolution is sufficient. It is therefore necessary to arrange for a change at will of the number of revolutions to be timed.

In Fig. 2, which shows diagrammatically the arrangement of the timing system, the particular device for selecting the number of revolutions to be timed is the adding relay *AR*. It is similar to the Cenco interval timer, but sturdier, and has been modified by replacing its pointer and scale with a contact arm and commutator, *C*. The contact arm moves 1/100 of the circumference with each impulse through its coils. A hard rubber ring, containing fifty brass segments imbedded radially in it, forms the circumference against which travels the hardened steel roller at the end of the arm. The fifty segments are divided into three circuits. One contains every odd fifth; the second, every even fifth; and the third, all the other segments. Thus by joining all the three circuits together we get a contact every two impulses through the electromagnet; by joining only the first two, we get a contact every ten impulses; and by using either of the first two

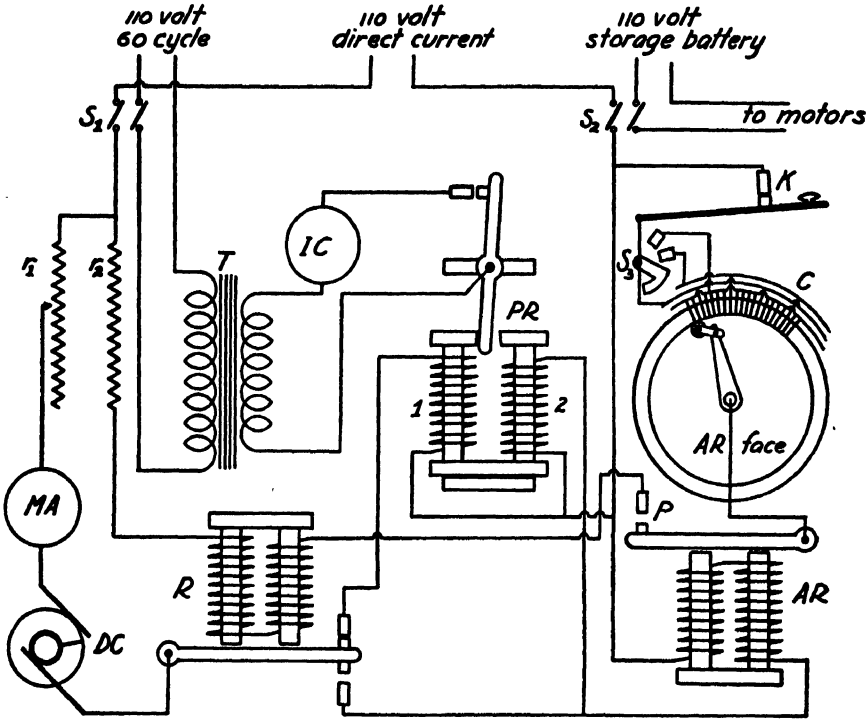

FIG. 2. Diagram of wiring and construction of the apparatus made to record automatically the speed of the sectored disc used to interrupt the light for critical frequency measurements. A complete description is in the text.

alone we get a contact every twenty impulses. The function of these contacts is to actuate an ordinary relay, *R*, which makes the electrical impulses generated by the sector disc control the opening or closing of the timing circuit through the polar relay *PR*. An additional contact *P* is put on the armature of the adding relay *AR*, to prevent arcing at the commutator *C*.

The complete system may be understood by first following the circuit through the contact *DC* on the sector disc shaft. Beginning at the 110 volt direct current supply, one side of the line comes through one pole of the switch, S_1, the variable resistance r_1, the milliameter *MA*, and the contact *DC* to the armature of the relay *R*. From there it can go through either one of two windings on the polar relay *PR* depending on whether there is current flowing through the coils of relay *R* or not.

If there is a current through the coils of *R*, then the circuit from *DC* will go into coil 1 of *PR* and out through switch S_2 to the other side of the line. Starting with the armature of the *PR* in an open position, impulses passing through coil 1 have no effect since only an impulse through coil 2 can close it.

When, however, there is no current through the coils of relay *R*, the circuit from *DC* divides into two parallel paths. One is through coil 2 of the polar relay *PR*, causing its armature circuit to be closed and thus starting the Cenco impulse counter, *IC*, recording the impulses of the sixty cycle current. The other path of the circuit from *DC* is through the coils of the adding relay *AR*, which records the number of impulses generated by the contact *DC* by moving its arm one division per impulse as already described.

The commutator circuit of *AR* is in series with the coils of *R* and its power supply. Thus the impulses generated by *DC* control the current in the coils of *R*, which in turn controls the recording of time by the Cenco impulse counter by means of *PR*. Switch S_3 may be set to record the time interval between 3, 11, or 21 consecutive impulses, which then corresponds to the time for 2, 10, or 20 complete rotations of the sector disc.

Due to the fact that the duration of the contact through *DC* varies with rotational speed, it is necessary to use a variable resistance, r_1, and a milliameter, *MA*, in series with it to regulate the current. The milliameter is a moving iron type of 750 milliampere range chosen because of its sluggish response. The resistance is adjusted so that the milliameter reads between 150 and 200 milliamperes. This value was found to be the optimum for all speeds of the sectored disc. The fixed resistance, r_2, merely acts as a current-limiting device for R.

To operate the system, switch S_2 is closed first, thus starting the motors which actuate the sector disc. The observer adjusts the speed of the motor to an approximately correct value and signals the recorder. The recorder then closes S_1 and adjusts r_1 to the correct value indicated by *MA*. The circuit through *R* is now closed, and therefore the impulses generated by *DC* pass through coil 1 of *PR*, leaving the Cenco counter circuit open. The observer, after adjusting the speed to the critical value, signals the recorder who then initiates a measurement. This is accomplished by opening the circuit through *R* by means of key *K*. As soon as the key is opened, the armature of relay *R* is pulled away by its spring and the next impulse from *DC* does two things at once. It goes through coil 2 of *PR* which closes the Cenco counter circuit and moves the arm of *AR* one division. After this happens, *K* is released since the commutator circuit is now open. The following impulses from *DC* have no further effect on the timing circuit, but they continue to move the arm of *AR* one division per impulse until it reaches the next "live" segment. Immediately after the last impulse passes, the armature of *AR* closes the circuit through *R* and the following impulse from *DC* passes through coil 1 of *PR* which opens the Cenco counter circuit. Thus the Cenco counter gives directly the time in 1/120 of a second that has elapsed during the rotations corresponding in number to the steps between two adjacent "live" segments in the armature *C*.

The whole system was checked for errors in time introduced by the various relays by causing a powerful double-throw snap switch to operate another Cenco interval timer simultaneously with the complete train of relays. In about two-thirds of the trials, the whole system was 1/120 of a second slower than the check counter, while in the rest of the trials no difference between the two counters was apparent.

V

Procedure

Before beginning work the subject became dark adapted by remaining in the dark room in which the measurements were made. For observations with the fovea, at least 15 minutes stay in the dark was given before operations were begun, which means about 25 minutes of dark adaptation before the first observation was recorded. Dark adaptation for the fovea is complete in much less time than this (Hecht, 1921). For measurements with the periphery of the eye at least three-quarters of an hour, and most often 1 hour of dark adaptation was given, before the first readings were made. This permitted complete dark adaptation. The measurements were always begun at the lowest illuminations, except in the special instances when only the higher frequencies of flicker were being investigated. Under these circumstances only a short period of adaptation was given.

The subject sat comfortably with his head in a chin-rest, and was optically separated from the rest of the dark room by a cubicle around his head. This cubicle was open at the back; but when necessary a cloth was thrown over it and over the shoulders of the observer to exclude all light except that which enters his eye through the observation pupil. Later, by proper screening of the light source and by encasing it in a metal housing, the last precaution became unnecessary even at the very lowest illuminations.

With each measurement the subject looked at the field for about a minute before beginning the setting. The manipulation preceding a decision required at least a minute, usually more, especially at the very low illuminations. Thus by the time the setting was made, the eye had been observing the field for at least 2 minutes, and usually for much longer. This insured the adaptation of the functional retinal region to the intensity under investigation (Lythgoe and Tansley, 1929).

The measurements were made in one of two ways, either by finding the intensity required for the extinction of a predetermined frequency of flicker, or by finding the frequency of flicker which would just be extinguished at a predetermined intensity of illumination. For the first method the motor was turned on and regulated until it ran at a chosen constant speed. The subject then got ready, and the light was turned on. The subject controlled the wedge which he now adjusted until the flicker in the center of the field disappeared. The speed of the sector disc was immediately recorded, as well as the position of the wedge. The position of the wedge was then changed, and after a moment's rest, the subject again set it so as to extinguish the flicker. The speed of the sectored disc and the position of the wedge were again recorded. When the required number of settings had been made for one frequency, the subject rested for about 5 minutes in the dark, while the motor was changed and regulated for a different frequency of flicker. The settings were then made as before, and the procedure repeated until the selected frequencies had been investigated. This method was used only for the lower intensities, particularly with C. D. V. as subject.

For the second method, the wedge and filters were set for a given illumination, and by moving a sliding rheostat, the subject adjusted the speed of the rotating sector disc until flicker just disappeared. The remainder of the procedure was the same as in the first method. The second method was always used at the higher illuminations; in the later measurements with S. H. as subject, it was most commonly used for the lower illuminations as well.

We could detect no difference in the results secured by the two methods, and we therefore used them to their best advantage. For example, as will become apparent in the following paper, the critical fusion frequency for certain parts of the retina remains constant or varies but slightly over a large range of intensities. Obviously here it is better to keep the illumination constant and to vary the motor speed.

In the early measurements, especially with C. D. V., we made at least 5 and often 10 and 15 settings for a given frequency of flicker. As the subject gained in experience, and we gained confidence in the measurements, this number was reduced until we were satisfied with two settings if they agreed with each other. We found no difference in accuracy when securing two readings only as opposed to ten or more. In fact, especially with S. H., we found fewer readings much more desirable, since a complete set of readings over the whole intensity range could be made at one sitting without any feeling of fatigue or strain.

VI

SUMMARY

An apparatus and a procedure are described to measure the critical frequency of flicker using different portions of the eye. The observer, looking through a pupil of fixed dimensions, views a field of 2° whose illumination is periodically interrupted and which is surrounded by a field of 10° whose illumination is continuous but otherwise identical with the interrupted field. Various parts of the apparatus are concerned with controlling and recording the retinal position of the field, its intensity, its spectral composition, and the frequency of interruption of its illumination. The procedure is so simplified and regulated that a complete set of readings over the whole intensity range of vision can be made at one sitting without fatigue or strain.

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INTERMITTENT STIMULATION BY LIGHT

III. THE RELATION BETWEEN INTENSITY AND CRITICAL FUSION FREQUENCY FOR DIFFERENT RETINAL LOCATIONS*

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I

Present State of Problem

1. Previous Work.—The critical frequency at which the visual fusion of rhythmically produced illumination takes place depends for its value on a variety of factors. The most effective of these is the intensity of the illumination.

The dependence of critical frequency on illumination was recognized by Plateau a century ago (1829), and is apparent from the later work of Emsmann (1854) and of Nichols (1884); but it was Ferry (1892) who first proposed the formulation that the critical fusion frequency varies directly with the logarithm of the intensity.[1] Ferry's published measurements do not support his generalization. In a plot of critical frequency against log I, his data, though covering little more than one logarithmic unit, form a sharply curved line convex to the log *I* axis, and bear no resemblance to the results of later investigators.

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* A preliminary report of this work was given to the Dutch Ophthalmological Society in December, 1930, and appears in the *Nederl. Tijdschr. Geneesk.*, April 25, 1931, p. 2274. It was reported more fully at the meeting of the Optical Society of America in February, 1933, and appears in abstract in the *J. Opt. Soc. America*, 1933, **23,** 194.

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[1] Ferry's actual statement is that the "persistence" of vision is inversely proportional to log I. By "persistence" Ferry merely means the necessary duration of the light flash alone, at the critical fusion frequency. The interval is thus equal to one half of the reciprocal of the critical frequency. It was generally believed that this time interval measures "the duration of the retinal impression," and Ferry so construes his data. The term is still used by Allen (1926), though Grünbaum (1898) long ago exposed its absurdities.

Adequate measurements of the relation between intensity and fusion frequency were first made by Porter (1902) who used an intensity range of 1 to 50,000. His data fall on two straight lines intersecting at an illumination of about 0.25 meter candles. Porter's work was corroborated by Kennelly and Whiting (1907), by Ives (1912), and by Luckiesh (1914). Ives measured not only white light but also colored lights and found that the data for different parts of the spectrum show a dual logarithmic relation similar to that for white light. The slope of the lines, however, varies with the wave-length, the upper and lower lines varying independently. For blue light Ives found that the lower line becomes horizontal. All these peculiarities of slope disappear when a small field is used.

Allen (1919, 1926) has in general confirmed the work of Porter and of Ives, but has differed from them by drawing through his measurements about five short straight lines of different slope instead of the usual two. In our estimation, the data presented by Allen do not justify this treatment; the points appear to lie on a continuously curving line. The recent work of Lythgoe and Tansley (1929), distinctly gives no support to Allen's multiplicity of straight lines.

Lythgoe and Tansley's measurements confirm the logarithmic relation of intensity to fusion frequency, but Lythgoe and Tansley attach no importance to its strict formulation as done by Ferry, by Porter, and by Ives, and consider that their data agree only under certain conditions with the linear relation of critical frequency to log *I*. The same may be said about the measurements of Granit and Harper (1930), who found that for a range of about 1 to 1000 in intensity the critical frequency is very nearly directly proportional to the logarithm of the intensity. For higher intensities the relationship does not hold, and the curve of frequency against log *I* tends to become horizontal, as already found by Grünbaum (1898).

Recently Sälzle (1932) has measured this relation for the first time in an animal other than man. He finds for the dragon fly larva that critical frequency is a sigmoid function of log *I*, the curve being nearly horizontal at upper and lower critical frequencies. In a paper just published, Wolf (1933) records precisely similar measurements for the honey bee.

Of the other numerous observations relating to intensity and critical frequency, some cover so small a range (*e.g.* Piéron, 1922; Polikarpoff, 1926) that no certain conclusions can be drawn from them about these variables, while others deal with the influence of various conditions on critical frequency and are not relevant here (*cf.* Parsons, 1924).

2. Need for the Present Work.—In spite of all this work the relation between intensity and critical fusion frequency is not adequately known in several important respects. In the first place, none of the measurements cover a range of intensities sufficiently wide to define the relationship over the functional range of the eye, and to include very high and very low illuminations. As a result of this lack, we know almost nothing about fusion frequencies below 10 cycles and above 40 cycles per second.

In the second place, none of the measurements except those of Ives describe the real relation between illumination and retinal effect, because they were all made with the natural pupil, and thus contain an additional and uncertain variable. The correction of such data by means of existing measurements of the pupil area (Reeves, 1918), already a dubious procedure since Schroeder's (1926) work, has now become meaningless in terms of the studies on the pupil by Stiles and Crawford (1933).

In order that an adequate theoretical structure may be built for the physiology of intermittent illumination, it is obviously necessary to possess the data in a fairly complete condition. We therefore measured the relation between critical fusion frequency and intensity for different portions of the retina over as large a range of illumination as possible, and under such conditions as to render the data reproducible and definitive.

II

Method and Material

The details of the apparatus and of the procedure which we used for this work have, for editorial convenience, been described separately in the preceding paper of this group.

All the measurements here recorded were made with the right eye of C. D. V. and with the right eye of S. H. When C. D. V. was the observer, S. H. acted as manipulator and recorder. When S. H. was observer, the manipulations and recording were made in the main by Mr.

Morton Schweitzer, and occasionally by Mr. Simon Shlaer. We wish to record here our indebtedness to both these gentlemen for their kindness.

III

Measurements with the Fovea

The data which we secured fall into several groups, depending on the ideas which urged us to make them. The original measurements of Porter, when plotted as critical frequency against log *I* show two straight lines, one of small slope, and continuing from it, another of greater slope. In conformity with the duplicity theory (von Kries, 1929) it is generally supposed that the lower line represents the functioning of the rods, while the upper, steeper line represents the function of the cones. The transition from the dominance of one system to that of the other then corresponds to the region of intersection of the two straight lines, which in Porter's data comes at a frequency of about 18 cycles per second.

If this separation of rod and cone function is correct, it should be possible to get a more complete cone curve below this critical value by deliberately confining the measurements to the rod-free area of the fovea, and by maintaining the fixation at this place even below the break when the fixation normally would wander to the periphery. Our first measurements were therefore made with strictly central fixation. We used white light, and a flickering area 2° in diameter surrounded as already described by a 10° field continuously illuminated. The measurements thus concern that part of the fovea which according to Wolfrum (Dieter, 1924) is practically rod-free.

The lowest intensity at which readings can be taken in this manner with the fovea is obviously well above the threshold of the rod system. It is even above the thresholds of some of the foveal cones as well, because we had to choose such an intensity that the slowest interruption in the illumination was clearly visible with central fixation. This is very nearly 0.01 photons. Below these intensities the field appears uniformly illuminated with central fixation even when the central test area of 2° is completely extinguished.

The measurements for central fixation were taken over a period of 3 months for C. D. V. and of a year and a half for S. H. Fig. 1 shows the 176 individual measurements made by S. H. Each setting is separately recorded so as to give an idea of the reproducibility of the observations. In this respect, the measurements of C. D. V. are exactly like those of S. H., but about three times as numerous, and thus more difficult to plot similarly in one figure. It is clear that the measurements, though protracted over a long period of time, are concordant and describe a real relationship in the eye. They may therefore be averaged in groups to record this relationship. The data so averaged are given in Table I and in Figs. 2 and 3. The line drawn through the unaveraged, individual measurements in Fig. 1 is the same as the one drawn through the average data of Fig. 2, and shows that the process of averaging has merely served to smooth the data without in the least distorting the relationship which they describe.

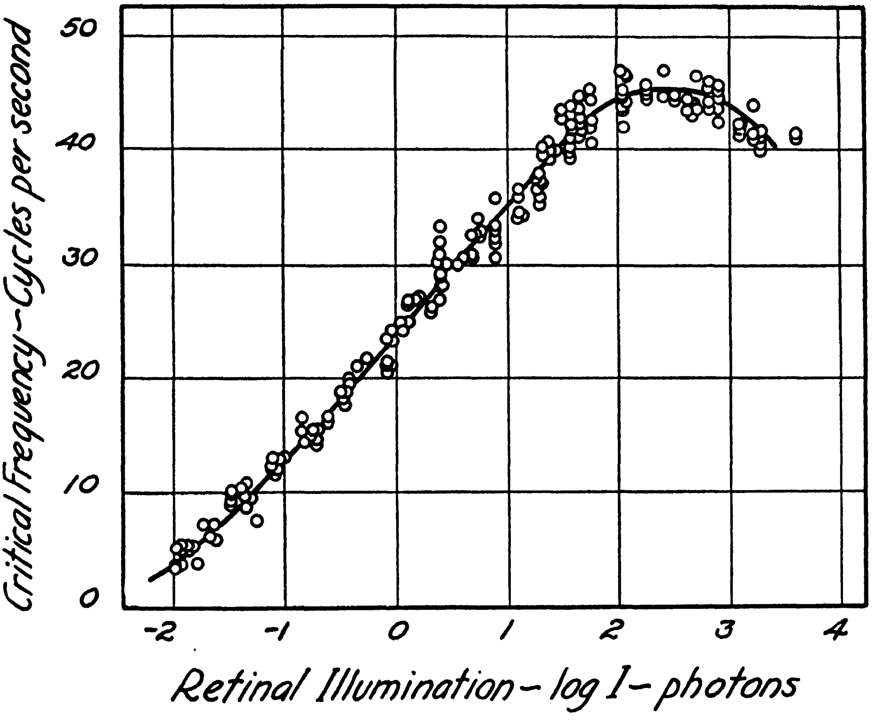

FIG. 1. Critical frequency function of the rod-free fovea as influenced by the illumination. Data for S. H. recording the 176 separate measurements. The curve is the same one as drawn through the average data in Fig. 2.

The data indicate that the direct logarithmic relation between intensity and critical frequency holds for the middle region of intensities, but that the complete relationship is more nearly sigmoid, the S shape being quite drawn out. Two aspects of the data require special consideration: first, the slope of the middle portion and second, the levelling-off and decrease of the critical frequency at the highest illuminations.

TABLE I

Critical fusion frequency (cycles per second) for white light at various retinal illuminations (photons). Test field 2° in center of fovea. Surround 10°.

	Right eye S.H.			Right eye C.D.V.		
Criterion	No. of readings	Retinal illumination	Critical frequency	No. of readings	Retinal illumination	Critical frequency
Normal	10	0.0131	4.59	30	0.0105	3.95
	4	0.0219	6.75	22	0.0207	6.14
	10	0.0424	9.59	21	0.0328	8.51
	8	0.0834	12.55	35	0.0635	11.70
	10	0.184	15.55	25	0.161	15.00
	8	0.391	19.63	23	0.440	19.86
	12	1.02	24.39	32	1.34	25.82
	14	2.43	29.18	33	7.18	33.98
	15	5.79	32.15	29	19.8	38.64
	20	18.0	37.64	40	56.8	44.13
	20	42.7	42.43	48	129.	47.38
	13	124.	44.96	35	334.	50.40
	10	321.	44.70	35	698.	52.87
	9	638.	44.68	22	1803.	52.00
	13	1832.	41.66	25	3556.	52.15
				28	6039.	51.03
No flicker on slight shift	10	490.	50.3			
	5	1585.	51.3			
	12	4571.	48.0			
Rapid readings	5	120.	41.7			
	3	331.	40.7			
	2	1000.	39.4			
	3	1950.	37.5			

In the range of intensities between about 0.1 photons and 100 photons, the data, when plotted as in Figs. 2 and 3, lie with extra-

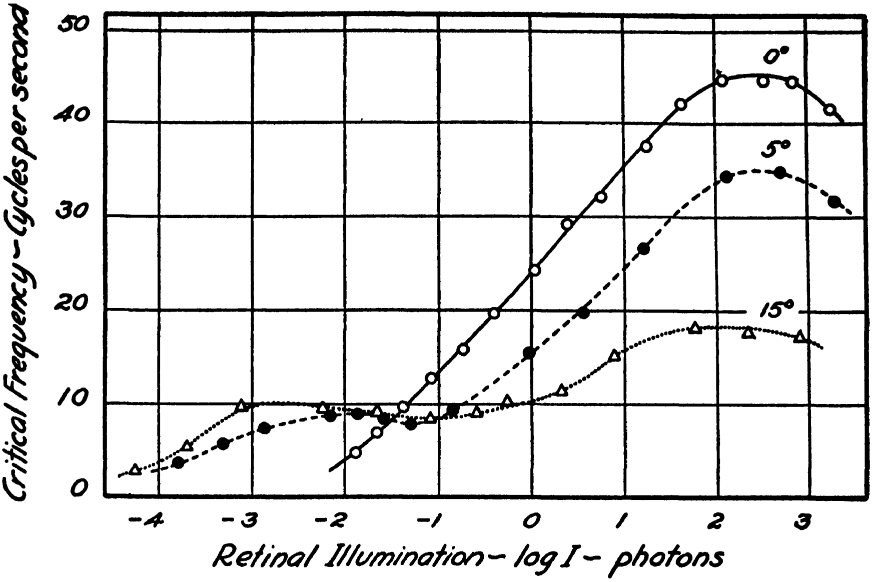

FIG. 2. Data for S. H. showing relation between critical frequency and log I for white light for three different retinal locations: at the fovea, and at 5° and 15° above the fovea.

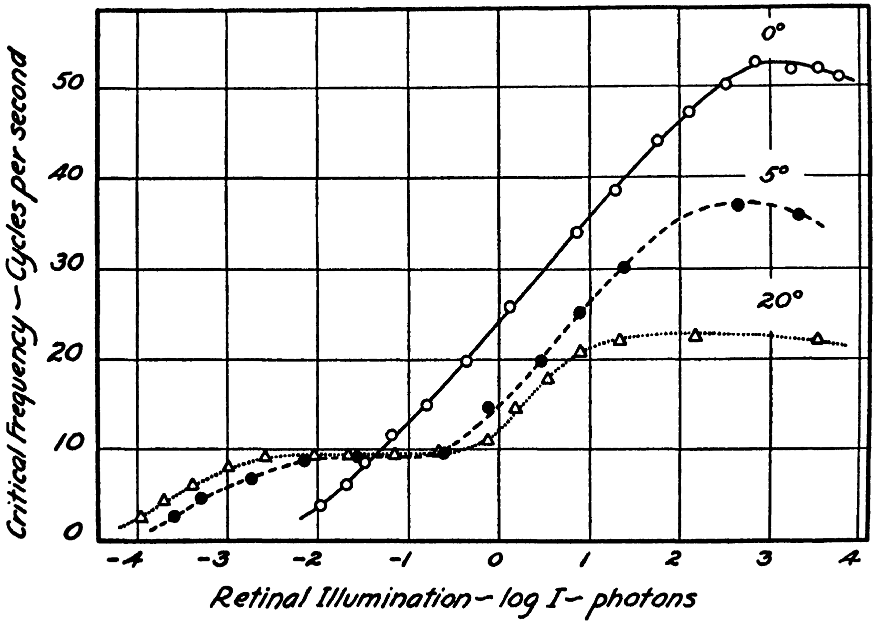

FIG. 3. Data for C. D. V. showing relation between critical frequency and log I for white light for three retinal locations: at the fovea, and at 5° and 20° above the fovea.

ordinary precision on a straight line. In this respect we can confirm Porter, Ives, and the other workers. The slope of this line is 11.1 for C. D. V., and 11.0 for S. H.

We may strictly compare our measurements with those of Ives (1912 and 1922), the only previous worker who used a pupil of fixed dimensions. The slope of Ives' 1912 data is 11.2, whereas for his 1922 data it is 10.0. The slope of the upper portion of Porter's data is 12.4. Kennelly and Whiting's slope is 11.0. Luckiesh's slope as published is 5.6, which is an extraordinarily low value. Most sectored wheels are constructed to give 4 cycles per revolution, and it is quite possible that Luckiesh erred in multiplying his motor frequencies by 2 and not by 4 to give cycles per second. His published slope, when multiplied by 2 gives 11.2. The same holds for Granit and Harper whose slope appears to be 5.5, but whose values must clearly be multiplied by 2 to record cycles per second and not motor frequencies. Their slope is therefore 11.0. Lythgoe and Tansley's data for the fovea contain 3 or 4 points in this region of the curve. For their only observer who was trained in visual work (R. L.) these points are regular and show a slope of 11.0. For their two untrained observers the points are irregular; but they seem to show a slope of about 9.0. Allen's (1926) measurements give a slope of 8.6 for yellow light of 570 mμ, which on the basis of general experience, may be considered the same as for white light. Thus most observers record values between 9 and 12, with a preponderance of 11. These variations do not seem to be connected with any obvious experimental conditions like pupil area, binocular observation, or size of field.[2]

Little need be said about the data below 0.1 photons. The critical frequency continues to decrease as log I decreases forming a gentle curve convex to the axis of abscissas, and stopping fairly abruptly when with central fixation the field appears uniform even when the test area is extinguished.

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[2] The measurements with white light here recorded were terminated for C. D. V. in 1930 and for S. H. early in 1932. Recently, *i.e.* about a year after the series was terminated, measurements with white light, with foveal fixation, and with the identical apparatus have been made by S. H. with the startling result that the slope of the data is now nearly 10.0 instead of 11.0. It is significant also that Ives in later publications (Ives, 1922) shows a similar change in slope from 11.2 to 10.0 for white light. Obviously there are unknown factors which seem to influence the value of the slope.

At the highest intensities the relation between critical frequency and log *I* rapidly ceases to be linear. As the intensity is raised a maximum critical frequency is soon reached, beyond which a further increase in intensity results in no further increase in critical frequency; rather it results in a decrease. The maximum critical frequency comes at about 500 photons for S. H. and at about 1000 photons for C. D. V. The value of the critical frequency at this maximum is 53 cycles per second for C. D. V. and 45 cycles per second for S. H. With a further increase in the intensity, the critical frequency distinctly decreases. At first we were skeptical about this, and therefore made many measurements in order to be certain of it.

In the course of these observations at the higher intensities we tried two variations in the technic for securing the data already given. In the first, the procedure was like that heretofore used, except that the end-point for the extinction of flicker was considered reached on prolonged observation only when no flicker was apparent even on a slight shift in fixation. The data, shown in Table I, indicate that by this rather undesirable criterion the critical frequency is raised considerably. In the second procedure, rigid fixation was maintained as usual, but the readings were made as rapidly as possible, say in about 30 seconds, thus preventing the complete adaptation of the eye to the experimental intensity. The data, also given in Table I, show clearly that inadequate light-adaptation decreases the critical frequency, a fact already evident from the work of Lythgoe and Tansley. The significant thing about these data, is that in common with the procedure normally used, they show a maximum critical frequency and a decline at the highest intensities.

Considered as a whole, the foveal measurements definitely bear out the general notion advanced to account for the abrupt change in slope in the original data of Porter and in the subsequent measurements of Ives. This is that the steeper part of the data represents the function of the cones, and the less steep part represents the participation of the rods. When, as has been done by us, the measurements are confined to the fovea, in an area which is practically rod-free, only one continuous relationship appears between critical frequency and intensity over the whole range, and it is to this relationship that the cone portion of previous investigators clearly corresponds.

IV

Measurements with the Periphery

The correctness of this conclusion becomes even more apparent when the measurements are made with regions of the retina outside the fovea centralis. We measured the critical frequency for white light with the same set-up as before but with fixation at 5° above the center, 15° above the center, and 20° above the center. The data thus concern a retinal test area of 2° diameter having a surround of 10°, and situated at 5°, 15°, and 20° above the center of the eye.

TABLE II

Critical fusion frequency (cycles per second) for white light at various retinal illuminations (photons). Test field 2° placed 5° above center of fovea. Surround 10°.

Right eye S.H.			Right eye C.D.V.		
No. of readings	Retinal illumination	Critical frequency	No. of readings	Retinal illumination	Critical frequency
4	0.000166	3.50	11	0.000258	2.53
2	0.000491	5.64	9	0.000518	4.59
4	0.00134	7.31	7	0.00185	6.80
4	0.00710	8.51	5	0.00698	8.90
2	0.0138	8.97	12	0.0276	9.21
5	0.0264	8.25	12	0.239	9.67
4	0.0514	7.70	12	0.764	14.80
6	0.146	9.41	7	2.98	19.90
6	0.968	15.32	10	7.93	25.20
6	3.66	19.70	13	24.2	30.16
7	16.5	26.66	11	448.	37.10
4	138.	34.70	8	2118.	36.00
5	514.	34.96			
5	1954.	31.70			

The data for 5° above the center are given in Table II and Fig. 2 for S. H. and in Table II and Fig. 3 for C. D. V. In all essentials the two sets of measurements agree. At the lowest illumination the critical frequency rises very distinctly with log I. As the intensity reaches about 0.01 photons, the critical frequency ceases to increase, and remains approximately constant over a range of 1.25 logarithmic units.

For C. D. V. this plateau is horizontal within the accuracy of the measurements; for S. H. the plateau has a slight, but distinct undulation. The undulation is not a product of averaging the data. It appears in every set of measurements made by S. H., and occasionally in those of C. D. V. Examination of the data of Ives shows the presence of such an undulation in the measurements for blue light at lower intensities, where Ives supposes the relation between critical frequency and log *I* to be horizontal; it is also apparent in some of the data of Lythgoe and Tansley.

The plateau in our data continues till about 0.2 photons, after which the critical frequency rises with log *I*. It continues to rise until it reaches a maximum at about 400 photons, after which it decreases as the intensity increases.

Figs. 2 and 3 show that the data for 5° off-center clearly fall into two parts. The first is at low intensities, where the critical frequency first rises with log *I* and then reaches a maximum which is approximately maintained. The intensity range covered by this rise and plateau is about 3.25 logarithmic units. The second part also begins with a rise in critical frequency as log *I* increases, and also terminates when the critical frequency reaches a maximum, and then declines. The intensity range covered by the second part is about 4 logarithmic units.

For the low intensity rise of critical frequency, the slope of the data is 5.0 for C. D. V., and 4.5 for S. H. There are only three points each available for these determinations, but the points are well established. For the high intensity rise in critical frequency the slope of the data is 10.5 for C. D. V. and 8.5 for S. H. The slope for C. D. V. is thus only slightly less than for the fovea, whereas for S. H. it is distinctly less for the 5° fixation than for central fixation.

The only measurements with which we can compare ours are those by Lythgoe and Tansley, who made a special point of determining the slope of their data at the higher illuminations for a 1° field placed 10° peripherally. Their two observers give slopes of 14.4 and 11.7 respectively. Lythgoe and Tansley state that the slope remains the same for all parts of the retina, and indeed give the slope for one observer at 50° off-center as 11.7. Their other data (*cf.* especially their Fig. 12) distinctly do not bear out this conclusion, but indicate instead a higher slope for the periphery than for the fovea. This difference between their and our data may be due to the difference in size of surround: ours covered 10° whereas theirs covered the whole eye. This may account also for the rather high values of the critical frequency found by them for the periphery.

TABLE III

Critical fusion frequency (cycles per second) for white light at various retinal illuminations (photons). Test field 2° placed 15° above center of fovea for S. H. and 20° above center for C. D. V. Surround 10°.

Right eye S.H. 15° above center			Right eye C.D.V. 20° above center		
No. of readings	Retinal illumination	Critical frequency	No. of readings	Retinal illumination	Critical frequency
2	0.0000551	2.62	9	0.000109	2.65
3	0.000205	5.33	9	0.000194	4.44
2	0.000760	9.61	9	0.000402	6..08
2	0.00577	9.31	9	0.00104	8.10
2	0.0214	9.14	8	0.00258	9.04
2	0.0796	8.43	8	0.00887	9.26
2	0.551	10.15	7	0.0209	9.44
2	2.05	11.45	9	0.0678	9.63
2	7.60	15.05	9	0.218	9.81
2	57.7	18.05	9	0.726	11.30
3	214.	17.63	9	1.52	14.83
2	796.	17.05	9	3.24	18.00
			5	7.78	21.00
			15	21.1	22.06
			13	144.	22.60
			9	3319.	22.10

The new element contained in our measurements of the periphery is the existence of two separate parts to the relationship between critical frequency and intensity. The measurements with the test field farther out in the periphery confirm and extend these findings. The data for a retinal test area of 2° with a 10° surround placed at 15° above the center are given in Table III and Fig. 2. The data for a similar area placed at 20° above the center are given in Table III and

Fig. 3. The data show the same division into two parts, each with a rise of critical frequency versus log I and subsequent plateau as do the data already given for a 5° peripheral displacement.

The slope of the rise at low intensities is 6.1 for C. D. V. and 6.0 for S. H. The slope for the rise at the higher intensities is 9.6 for C. D. V. and 7.0 for S. H. Again the value for C. D. V. is not very much below that for the fovea, whereas that for S. H. is distinctly less.

The plateau for the 15° and 20° off-center measurements is about 0.75 log units longer than for the 5° off-center data. This is because at 15° and 20° the low values of the critical frequency occur at lower intensities and the high values occur at higher intensities than at 5° off-center. We are quite certain of this broadening out of the curve at low and high intensities for the more peripheral positions because we made special measurements to test this point. We do not record these special measurements here because they merely corroborate those already given in the tables and figures.

V

Various Quadrants

The results we secured with peripheral stimulation seemed so striking, and yet so clear in their significance that we wished to be certain of their general validity over the retina. The peripheral data so far reported deal with regions above the fovea. We therefore measured the relation between critical frequency and illumination for the same test area and surround as before, but placed 5° peripherally in the four principal directions: up, down, nasal, temporal.

The data for C. D. V. are given in Table IV and in Fig. 4. Each group represents only one set of measurements made in a day. Each point is thus the average of two or three concordant readings. It is apparent that the essential phenomenon recorded is a general one, since the data all show the same division into two parts, with a rise and a plateau for each part.

Certain details are to be noted in which the four directions differ. The height of the low intensity plateau seems to increase in the following order: temporal, nasal, down, and up. The two horizontal positions have the same slope for the high intensity rise; its value is 8.7, and it is therefore less than the up position which as before is 10.4.

TABLE IV

Critical fusion frequency (cycles per second) for white light at various retinal illuminations (photons). Test field 2° placed 5° away from center in four different directions. Right eye of C. D. V. Surround 10°.

Nasal		Temporal		Down		Up	
Retinal illumination	Critical frequency	Retinal illumination	Critical frequency	Retinal illumination	Critical frequency	Retinal illumination	Critical frequency
0.000372	2.20	0.000372	2.24	0.000438	1.93	0.000310	2.50
0.000873	4.24	0.00163	4.84	0.000693	4.08	0.000647	4.24
0.00381	6.88	0.00604	5.76	0.00145	5.80	0.00214	6.32
0.00980	6.96	0.0219	6.68	0.00418	7.40	0.0163	8.92
0.0276	7.48	0.0662	6.88	0.0142	7.84	0.0235	8.28
0.0662	7.40	0.200	8.68	0.0428	7.96	0.289	10.4
0.103	7.40	0.317	10.7	0.129	8.28	0.491	12.4
0.159	9.08	0.873	14.8	0.390	10.5	1.38	16.8
1.05	15.2	3.99	19.9	0.693	15.2	6.18	24.2
4.80	20.3	15.5	25.0	1.59	19.7	19.5	29.4
38.1	29.6	56.4	30.3	5.02	23.8	28.3	29.8
276.	35.1	276.	36.2	15.2	30.3	1589.	34.8
				41.8	36.4		
				276.	37.7		
				1026.	37.4		
				4375.	36.0		

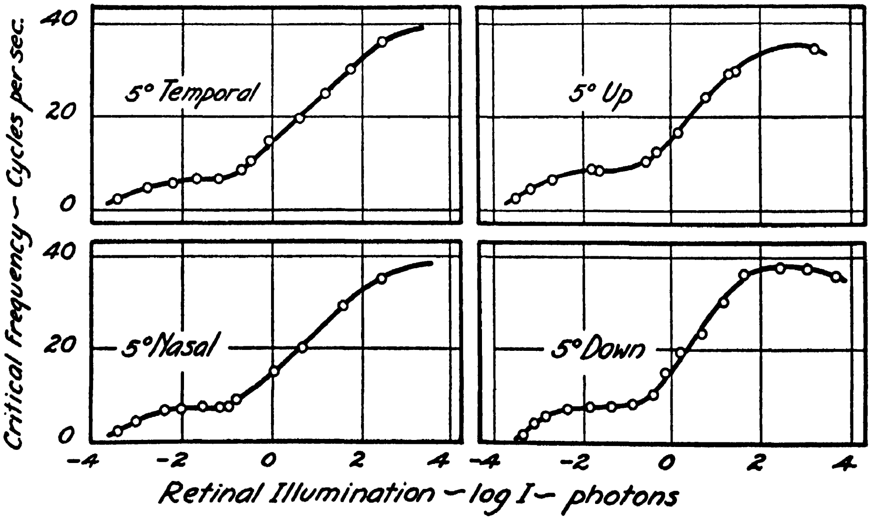

FIG. 4. Data for C. D. V. showing relation between critical frequency and log *I* for white light for 5° off-center in the four principal retinal directions.

Moreover the down position data show a slope of 12.0 which is distinctly greater than that of the up position and of the fovea. Very likely these variations in detail represent variations in structure at which we can only guess in our present knowledge. Possibly they are related to the population density of the elements in various parts of the retina.

VI

Structural Interpretation

The general relations among the data are apparent when they are considered all together as in Figs. 2 and 3. Their interpretation in terms of the well known histological composition of the retina is immediately obvious.

The data for the fovea are represented by a single relationship between critical frequency and illumination. Since there are almost no rods in the foveal area used for the measurements, the central fixation data must surely record the behavior of the cones of the fovea.

The data for the periphery are represented by two separate relationships between critical frequency and illumination. The part at the higher illuminations resembles the foveal curve in appearance, and for the 5° eccentric field, has practically the same slope. Clearly this portion also represents the behavior of the cones. Moreover, the portion of the peripheral data at low illumination is apparently a distinct and complete relationship, and does not appear in the foveal curves. The obvious conclusion here is that the rise and the plateau at low illuminations represent the function of the rods.

These conclusions are strengthened by the fact that as the measurements are made farther in the periphery, the low intensity plateau becomes longer, and therefore the separation between the rise at low intensities and the rise at high intensities becomes greater. Thus the rod system becomes more sensitive and the cone system less sensitive as the measuring area moves from the center farther into the periphery. This is in keeping with the anatomical increase in number of rods and the converse decrease in cones as one proceeds along the retina toward the periphery.

All our data and their structural interpretation are thus strictly in line with the knowledge and ideas embodied in the duplicity theory (von Kries, 1929) which functionally separates the anatomically distinct rods and cones, and places the dominance of the rod system at low illuminations and the dominance of the cone system at higher illuminations. It will be shown in a later paper of this series how this description of the data is further borne out by work with colored lights (*cf.* Hecht and Verrijp, 1933).

VII

SUMMARY

When measurements of the critical fusion frequency for white light over a large range of intensities are made with the rod-free area of the fovea, the relation between critical frequency and log I is given by a single sigmoid curve, the middle portion of which approximates a straight line whose slope is 11.0. This single relation must be a function of the foveal cones.

When the measurements are made with a retinal area placed 5° from the fovea, and therefore containing both rods and cones, the relation between critical frequency and log I shows two clearly separated sections. At the lower intensities the relation is sigmoid and reaches an upper level at about 10 cycles per second, which is maintained for 1.25 log units, and is followed by another sigmoid relationship at the higher intensities similar to the one given by the rod-free area alone.

These two parts of the data are obviously separate functions of the rods at low intensities and of the cones at high intensities. This is further borne out by similar measurements made with retinal areas 15° and 20° from the fovea where the ratio of rods to cones is anatomically greater than at 5°. The two sections of the data come out farther apart on the intensity scale, the rod portion being at lower intensities and the cone portion at higher intensities than at 5°.

The general form of the relation between critical frequency and intensity is therefore determined by the relative predominance of the cones and the rods in the retinal area used for the measurements.

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INTERMITTENT STIMULATION BY LIGHT

IV. A THEORETICAL INTERPRETATION OF THE QUANTITATIVE DATA OF FLICKER

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I

The Essence and Location of Flicker Changes

In the intermittent stimulation by light the outside agent alternates abruptly between zero and the particular maximal intensity. When the frequency of this alternation is sufficiently low, the difference between these extreme conditions is completely perceptible in sensation: the brightness during the light period is maximal, and during the dark period it is zero. As the alternation frequency increases, the two sensations become less sharply delimited in time and less clearly separated in intensity: the light period loses in brightness, and the dark period gains in brightness. The more frequent the alternation, the less is the difference between the successive sensations; and when the frequency is sufficiently high the difference between the successive sensations vanishes so that the outside fluctuating light appears continuous. This shows that it is the time factor which is important in the transformation of external physical discontinuity into internal sensory continuity.

The three places where this transformation may conceivably take place are the retina, the conducting paths, and the brain. Of these, the conducting mechanism may be quickly eliminated. All the modern work shows that if an optic nerve were supplied with a group of impulses sharply separated from, and rapidly alternating with a period of no impulses, it could maintain the separation of the two periods even at a frequency of 50 cycles per second. However, in flicker the optic nerve receives no such sharply delimited alternation of phases. Adrian and Matthews (1928) found that in the eel's eye the impulses along the nerve show no periodic changes for a frequency of intermittent retinal illumination as little as 5 cycles per second at low intensities, even though the nerve carries groups of impulses occurring three times as frequently at higher illuminations. Thus while the brain may be responsible for a part of the transformation of intermittent illumination into continuous sensation, it is certain that most of this transformation has already been accomplished in the retina.

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This conclusion is supported by other considerations. Brightness is probably determined by the number of impulses per unit time which reach the brain from a group of related retinal elements. Differences in the sensation of brightness can therefore be produced by differences in the number of elements which function in a given group and by differences in the frequency of impulses coming from each continuously functioning element.

If the determining factor is the number of functional elements, recognition of flicker means such a fluctuation in the condition of the retina that during the light and dark phases of the flicker cycle a critical number of elements of marginal thresholds in a given group alternately function and cease to function. Flicker then disappears when the amplitude of fluctuation is below that required to set off and on these elements of marginal threshold. A similar condition obtains if the determining factor is the frequency with which a group of elements discharge impulses to the brain. In either case the controlling influence is in the retina.

II

The Data of Flicker

Before considering the possible events in the retina which may be concerned in flicker we wish to summarize the essential facts in this field. This is necessary in order to lay down the minimal requirements for any theoretical treatment of the subject.

The first fact concerns the physiological effectiveness of intermittent stimulation by light when the frequency is above the critical frequency, that is, when flicker has disappeared. This is described by Talbot's law (Talbot, 1834) which states that the brightness under these circumstances equals the original steady brightness multiplied by the fraction which the actual duration of illumination is of the total duration of a complete cycle of light and dark. In other words, a reduction in the time of action of the light is equivalent physiologically to a corresponding reduction in its intensity. The evidence has been presented in the first paper of this series (Hecht and Wolf, 1932) and is derived from a sufficient variety of sources and animals to render certain the validity of Talbot's law.

The second group of facts deals with the influence of the intensity of illumination on the critical frequency. The details have been presented in the preceding paper of this series. The relation between critical frequency and log I is sigmoid in form for both rods and cones. The maximum critical frequency for the cones is about 50 cycles per second; that of the rods about 10 cycles per second. The slope of the central part of the relationship is about 11 for the foveal cones and about half as much for the rods. The slope for the peripheral cones is about the same as for the foveal cones, but seems to vary for different persons and for different retinal positions. Also for a given illumination the critical frequency depends on the stimulated retinal area (Granit and Harper, 1930).

The third set of data are concerned with the relation of critical frequency to the condition of the eye. Schaternikoff (1902) found that the critical frequency declines regularly and concomitantly with the dark adaptation of the eye. This has been confirmed by Lythgoe and Tansley (1929) who showed in addition that the critical frequency rises during light adaptation.

The fourth fact, found by Porter (1902) and corroborated by Ives (1922), is that for a given illumination the critical frequency is a maximum when the light and dark periods are approximately equal, and declines as either becomes significantly longer than the other.

III

Previous Formulations

No existing theoretical treatment supplies a synthesis of these facts. The ideas of Fick (1863), S. Exner (1870), and K. Exner (1870), though adequate in themselves, were concerned only with Talbot's law and antedate the knowledge contributed by Porter of the relation between critical frequency and log *I*. Troland's formulation (1913) is probably sound in that it uses the idea of a reversible sensory system. However it is too general to be of use with a set of data. Lasareff's derivation (1926) also relies on a reversible sensory system and furnishes a description of Schaternikoff's data on the influence of dark adaptation, and of the usual logarithmic relation between intensity and critical frequency. Lasareff's treatment omits Talbot's law, as well as the relation between light and dark ratio and frequency, and we have been unable to understand it sufficiently well to test its application to these properties of flicker.

Ives' "theory of intermittent vision" is the best and most concrete effort at including all the data existing at the time (1922). Ives assumes three steps in vision. The first is a reversible photochemical reaction of "such a nature that the equilibrium value under steady illumination is proportional to the logarithm of the stimulus;" the second step consists of a diffusion process according to the Fourier diffusion law, the purpose of which is to account for the conduction of the substance formed during the first step; the third step involves the perception process "in which the criterion for perception is that the time rate of change of the transmitted reaction must exceed a constant critical value." The fairly complex mathematical development of these ideas yields Talbot's law, the logarithmic relation, and the relation between light and dark ratio and critical frequency.

We find three difficulties in adopting Ives' treatment. First, Ives deals with the important logarithmic relation between intensity and photochemical effect merely by assuming it. Second, the logarithmic relation between intensity and critical frequency does not follow even from this assumed photochemical formation but results from the character of the diffusion of the photoproducts postulated in the second step. Ives, himself, realizes the difficulty of placing such major theoretical emphasis on this diffusion or conduction process. Third, even after accepting these two steps, one finds that the slope of the line relating log *I* and critical frequency does not come out from the theory, but is a value to be taken from the data.

IV

Photochemical Derivation

Because of these inadequacies in existing theory we propose to develop some ideas of our own with regard to flicker. The equations to be derived follow from the simple properties of the very first step in photoreception, and involve neither a special logarithmic assumption between intensity and photochemical effect, nor a second "conduction" process.

We wish explicitly to state that we consider our treatment only as a first approximation. The retina is a complicated structure, and not enough is known about it to furnish the material for an adequate formulation of so complex a phenomenon as flicker. However, no matter what else occurs in the retina, the very first step in vision must involve a photochemical change having certain fairly well defined characteristics (Hecht, 1931). Since the products of this photochemical change serve only to *start* the complicated train of events which finally yield a series of impulses in the optic nerve, it cannot be expected that the behavior of the photochemical system alone will yield a complete description of the receptor process. Nevertheless, because quantitative ideas are available in photochemistry it is necessary to discover how far this very first photochemical transformation can go in characterizing the photoreceptor process as a whole. In the present instance it is instructive and important to point out that the following study of this very first step in its relation to intermittent illumination already shows it to possess many of the essentially quantitative properties of the physiology of flicker.

We start with the familiar assumption (Hecht, 1931) that the first step in the photoreceptor process is a reversible photochemical reaction. The light changes a sensitive substance into two products which start the series of events ending in a nerve impulse, and which can recombine to form the original sensitive substance according to an ordinary "dark" reaction. Following well accepted photochemical ideas, the velocity of the reaction as a whole as it proceeds under the influence of light may be written

$$\frac{dx}{dt} = k_1 I(a - x) - k_2 x^2 \qquad (1)$$

where I is the intensity, a the initial concentration of sensitive material and x, the concentration of photoproducts. In the absence of light only the "dark" reaction goes, and the equation

$$\frac{dx}{dt} = k_2x^2 \tag{2}$$

gives the rate at which it forms the photosensitive material.

In intermittent illumination these two reactions alternate rapidly, and at the disappearance of flicker, they form a steady state in which what has been decomposed during the light period is regenerated during the dark period. The time occupied by each light or dark exposure is Δt, and is very short when flicker disappears. Therefore the velocity dx/dt may be considered constant, and equal to $\Delta x/\Delta t$, where Δx is the change in concentration of photoproducts occurring between the beginning and the end of each exposure. Since the light and dark periods in these experiments are of equal duration, these two velocities are equal. On equating them we get

$$\frac{KI}{2} = \frac{x^2}{a - x} \tag{3}$$

where $K = k_1/k_2$. This is Talbot's law for equal light and dark periods.

For the critical disappearance of flicker it is supposed that $\Delta x = c'$; that is, that the fluctuation Δx in the chemical concentration of photoproducts is constant at a value c' which is just too small to cause the physiological change corresponding to a perceptible change in sensation of brightness.[1] The critical frequency is n cycles of light and

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[1] This is a special form of the assumption which has run through much of the theoretical work with the photoreceptor process (Hecht, 1923, 1931). However, we have also tried a derivation which assumes that flicker disappears when Δx becomes less than a *constant fraction* of x. This is equivalent to writing that $\Delta x = h'x$, where h' is a constant. The remaining development is similar to the one given in the text. As above, $n = 1/2\Delta t$, which gives from equation (2) that $x = 2h'n/k_2$. This, when substituted in equation (3) yields

$$\frac{hKI}{2} = \frac{n^2}{ha-n} \tag{5a}$$

where $h = k_2/2h'$. Equation (5a) is obviously identical in form with equation dark flashes per second. Thus $n = \frac{1}{2}\Delta t$. Substituting these values of Δx and of Δt in equation (2) and putting $\sqrt{2c'/k_2} = c$, we get

$$x = c\sqrt{n} \tag{4}$$

as the relation between critical frequency and mean concentration of photoproducts in the reversible photochemical system. If now we put this value of x into equation (3) of the steady state the result is

$$\frac{KI}{2c} = \frac{n}{\frac{a}{c} - \sqrt{n}} \tag{5}$$

which should describe the dependence of critical frequency on intensity, in a single homogeneous system such as a rod or a cone. In equation (5) a as usual is put at 100 per cent, while K and c are constants to be found from the data themselves. It is apparent that c is 100 divided by the square root of the maximum value of n, while K determines the position of the data on the axis of abscissas.

V

Numerical Comparisons

Figs. 1 and 2 show the relation of equation (5) to the data of critical frequency secured by ourselves and presented in the preceding paper of this group. Consider first the measurements made with the rods located in the retinal area 5° above the center of the eye. For S. H., if we make $K = 1{,}400{,}000$ and $c = 33.9$, equation (5) becomes $20{,}600\ I = n/(2.95 - \sqrt{n})$, which is the line drawn through the rod data in Fig. 1. Similarly for C. D. V. if $K = 500{,}000$, and $c = 32.4$, then equation (5) becomes $7720\ I = n/(3.08 - \sqrt{n})$, which is the line through the rod data in Fig. 2. The adequacy of equation (5) as a description of the data shows that the flicker function of the rods behaves as if it were controlled by the initial photochemical process in photoreception.

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(3). We have applied it to the data and find that though not very different from equation (5), it is less adequate as a representation of the data even in a modification comparable to equation (6). For the lower intensities of the cones it yields values of n which are somewhat too high, more so than those derived from equations (5) and (6). Equation (5*a*) and its derivation is preferred by C. D. V., whereas S. H. prefers the one given in the text.

The flicker function of the cones, however, is not to be described quite so simply. The main difficulty concerns the slope of the middle portion of the data for which equation (5) yields a value too large by a factor of about 2. Considering the variation which this slope shows in the measurements for different retinal positions and the uncertainty of the precise structural factors which determine this variation, we have refrained from making any fundamental changes in the derivation of equation (5) for use with the cones. Instead we have adopted a purely arbitrary method of correcting for the slope by attaching an exponent α to the intensity I. Equation (5) then becomes

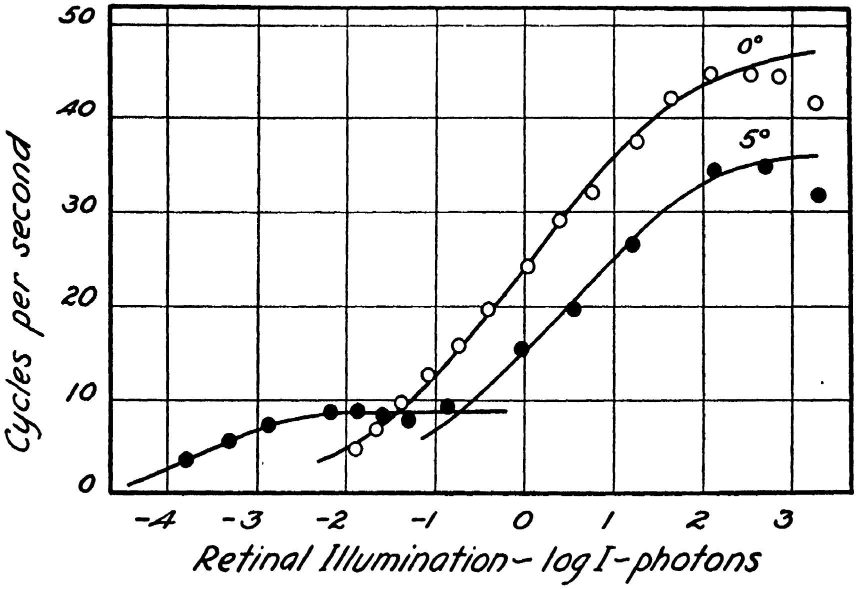

FIG. 1. Theoretical representation of data of critical frequency for S. H. given in the preceding paper of this series. The curve through the low intensity section of the 5° off-center data is drawn from equation (5) whereas the other two curves are drawn from equation (6). The specific values of the constants are given in the text.

$$\frac{KI^{\alpha}}{2c} = \frac{n}{\frac{a}{c} - \sqrt{n}} \tag{6}$$

which may now be used to compare with the data. We shall consider the possible meaning of α in a moment.

For the S. H. data of 5° off-center, if we put $K = 228$, $\alpha = 0.55$, and $c = 16.44$, then equation (6) becomes $6.93\ I^{0.55} = n/(6.08 - \sqrt{n})$ which is the curve drawn through the 5° cone data in Fig. 1. In the same way for C. D. V., if we put $K = 213$, $\alpha = 0.50$, and $c = 15.81$, then equation (6) becomes $6.74\ I^{0.50} = n/(6.33 - \sqrt{n})$ which is drawn through the 5° cone data in Fig. 2. For the foveal data of S. H. the curve in Fig. 1 has the equation $11.72\ I^{0.52} = n/(6.93 - \sqrt{n})$ derived by taking $K = 338$, $\alpha = 0.52$, and $c = 14.43$. For C. D. V. the curve through the foveal points in Fig. 2 has the equation $9.15\ I^{0.44} = n/(7.48 - \sqrt{n})$ secured by taking $K = 244$, $\alpha = 0.44$, and $c = 13.36$.

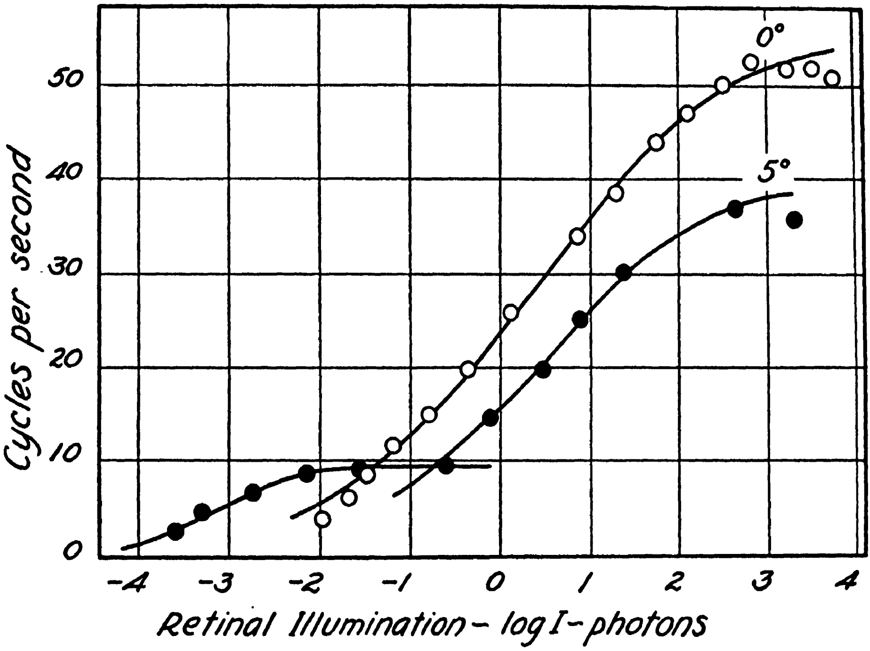

FIG. 2. Theoretical representation of data of critical frequency for C. D. V. taken from the preceding paper of this series. The curve through the low intensity section of the 5° off-center data is drawn from equation (5); the other two curves are drawn from equation (6). The specific values of the constants are given in the text.

We may therefore conclude from the numerical comparisons between the data and the theoretical derivation that the first step in photoreception considered as a reversible photochemical reaction is able to furnish a first order description of our flicker data.

VI

Deviations from Theory

There are two aspects in which the data appear to deviate from so simple a formulation. The first is the down-turn of the critical frequency at the very highest retinal illuminations. These are of almost blinding brightness, and work at such intensities frequently has the unpleasant consequence of headache and persistent after-images. Disturbances of function are therefore not unexpected and may have their origins in vascular or other changes not immediately relevant. It is for this reason that the cone theoretical curves in Figs. 1 and 2 have been made to terminate higher than do the data. Possibly also this down-turn is related to the processes which normally follow and are controlled by the photoproducts (Hecht, 1931), (Adrian and Matthews, 1927). Catenary systems of this kind tend to prolong the effects of the light period at the expense of the dark period, and at very high illuminations would require the frequency to be reduced in order that the dark period may become perceptible.

The second deviation from simple theory concerns the slope of the middle part for the cones, which is corrected by the exponent α. A possible explanation of the differences in the slope concerns the threshold distributions of the sensory elements and their connections (Lucas, 1905; Toy, 1922; Hecht, 1928). The photochemical derivation is for a homogeneous system, and applies to a given cell or to a number of cells of identical properties. However, if these cells and their connections, though activated by the same photochemical system, nevertheless possess different discharge thresholds, then their flicker function may require a new factor for its description.

Perhaps the simplest and most adequate explanation of the necessity for the exponent α derives from its numerical value. The values for α used here are of the order of 0.5, and could indeed have been made exactly equal to 0.5 without any significant deviation from the data. This means that the velocity of the photochemical action of light in the cones is a square root function of the intensity. Such a relation is fairly common in photochemical reactions, particularly in those involving the halogens (*cf.* Berthoud, 1926; and Griffith and McKeown, 1929). We have always assumed for the eye that the velocity is a linear function of the intensity of the light. This clearly holds for the rods; but there have already been indications in intensity discrimination data and also in visual acuity data (Hecht, 1931) that this is not adequate for the cones. It may be one of the contributions of the present data that they so clearly point toward the necessity of a square root function in this connection.

VII

Theoretical Extensions

It remains to consider whether the derivation here given accounts for the other characteristics of flicker previously enumerated. Talbot's law has already been dealt with in the first paper of this series (Hecht and Wolf, 1932) where its validity in terms of the photoreceptor system was demonstrated theoretically and experimentally.

The critical frequency falls during dark adaptation and rises with light adaptation (Lythgoe and Tansley, 1929). We omit a mathematical derivation of these results and merely point out how they follow from equations (1), (2), and (4). Equation (1) shows that during light adaptation the concentration of photoproducts increases, and equation (2) shows that during dark adaptation it decreases. Since according to equation (4) the critical frequency n is a function of the concentration x it follows that during light adaptation the critical frequency to a given light must increase, whereas during dark adaptation it must decrease.

The last aspect of flicker is the rise and fall of critical frequency at a given intensity as the ratio of dark period to light period increases. It is proposed to deal with this at some length at a future time; therefore only the barest outline need be given now. The general equation for Talbot's law (*cf.* Hecht and Wolf, 1932) is

$$\frac{KI}{p} = \frac{x^2}{a - x} \tag{7}$$

where $1/p$ is the fraction of the total cycle of light and dark occupied by the light period. Thus Δt is the duration of the light period,

$p\Delta t$ that of the whole cycle, and $(p - 1)\Delta t$ that of the dark period. The expression for the velocity during the dark period now becomes

$$\frac{\Delta x}{(p - 1)\Delta t} = k_2 x^2 \tag{8}$$

into which may be substituted the value of the critical frequency, $n = 1/p\Delta t$, and the previous assumption that $\Delta x = c$. The resulting relation between x and n is

$$x^2 = \frac{c}{k_2} \cdot \frac{p}{(p - 1)} n \tag{9}$$

and may now be substituted in equation (7). When solved, this yields

$$n = \frac{p - 1}{p^3} \cdot \frac{KIk_2}{2c} (KI + 2ap - \sqrt{4KIap + K^2I^2}) \tag{10}$$

as the relation between n, p, and I. At constant intensity this means that as p increases, the critical frequency n first increases to a maximum and then decreases. The relation which equation (10) gives is not quite symmetrical, but the data (*cf.* Ives and Kingsbury, 1916) are not unequivocal in this respect.

VIII

Comparisons with Other Visual Functions

The division of our flicker data into a rod component and a cone component each functional at a different intensity level is in keeping with the previous theoretical treatment of intensity discrimination, of visual acuity, and of dark adaptation (Hecht, 1931). The computations for intensity discrimination showed that the just effective difference in concentration of photoproducts required for a minimal intensity discrimination is much larger for the rods than for the cones. The same difference is shown in visual acuity, where the range of visual acuity units covered by the rods is much less than by the cones. Similarly here in the flicker data, the constant c in equations (5) and (6), which is proportional to the square root of the concentration change Δx, is more than twice as large for the rods as for the cones.

The value of Δx for the rods is thus more than four times as large for the rods as for the cones, which means that the capacity for flicker discrimination is much coarser in the rods than in the cones.

Another agreement concerns the relative values of K for the rods and for the cones. Both in intensity discrimination and in visual acuity, K for the rods is many times greater than K for the cones. The relation which this bears to the rate of dark adaptation of the two systems has already been discussed (Hecht, 1924). Here we find precisely the same difference in the K values, those for the rods being about a thousand times those for the cones.

It is not possible to make more than this roughly quantitative comparison of the constants and values secured from the various other data with those secured in flicker, because the two groups of data have been made under significantly different conditions. Our flicker data have been made with strict fixation and are always concerned with the behavior of specifically circumscribed retinal areas. The data of intensity discrimination and visual acuity, however, represent maximal functions of the eye as a whole in which the most effective region is used at any given moment. Thus at low intensities various parts of the periphery are used, while at higher intensities only the fovea is used. It would be of extraordinary interest in this connection to make measurements of visual acuity and of intensity discrimination with specifically fixated retinal areas.

IX

SUMMARY

A theoretical treatment of the data of intermittent stimulation by light is presented in terms of the familiar reversible photochemical system previously used for other properties of vision. It appears that such a system considered merely as the initial event in photoreception is capable of giving a first order quantitative description of the relation between critical frequency and illumination for different retinal regions, and of Talbot's law. Moreover the development of this concept shows that the general form of most of the existing relationships in flicker are already apparent in the characteristics of the behavior of this initial photochemical event.

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THE CONVERSION OF FAT TO CARBOHYDRATE IN THE GERMINATING CASTOR BEAN

I. THE RESPIRATORY METABOLISM

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The possible conversion of fat to carbohydrate in the mammalian body has been one of the most hotly contested problems in physiology. Nobody has yet brought forward evidence so convincing that his opponents on the other side of the argument have been compelled to yield ground. Very much of the so called evidence is worthless. It was with this conviction that the present writer persuaded certain of his pupils to take up the question and attempt to produce evidence which would be completely convincing, one way or the other. Three papers (1–3) already published have, it is believed, produced good evidence supporting the viewpoint of the late Professor Lusk, namely, that this conversion in the mammalian organism is at least extremely difficult and, under the conditions studied, not demonstrable.

From a careful reading of the literature on the germination of the fatty seeds, it appeared that in this instance the evidence, so far as it has been developed, favors the conception that fat is converted to carbohydrate (sugar) for the obvious purpose of increased diffusibility. At all events, this has been the interpretation of botanical physiologists. Since two lines of proof for conversion in the organs of the dog (and cat) had failed so signally under critical examination (2, 3) the writer wondered whether the evidence for the fatty seeds could really be so convincing as it seemed. It was with this attitude of skepticism that the present study was undertaken.

The problem has been approached from three directions: (1) the significance of the respiratory quotient during normal germination;

(2) the changing composition of the seed as shown by combustion; and (3) the nature of the chemical changes. Only the first division of the subject is presented in this paper; the others follow immediately in this issue.

HISTORICAL

A brief sketch of the historical background must suffice, as the subject has been adequately reviewed in several places (4–6). The original observation that in the germination of fatty seeds the volume of oxygen absorbed exceeds considerably that of the carbon dioxide given off, was made by de Saussure (7) and this observation gained special significance through the microchemical work of Sachs (8) confirmed by Schmidt (9) and the chemical analyses of Peters (10) and of Detmer (11) —all demonstrating that during the most active stage of germination fat disappears rapidly and carbohydrate takes its place. It appeared therefore, as Detmer and Godlewski (12) clearly recognized, that oxygen was being used for the production of carbohydrate, an oxygen-rich substance, from fat, an oxygen-poor substance, as well as for combustion of fat. Godlewski found the R.Q. for the period of most rapid absorption of oxygen to be between 0.55 and 0.65. He modifies the equations first proposed by Detmer for the transformation of triolein to starch after hydrolysis to oleic acid and glycerine. Thus:

$$(C_{18}H_{33}O_2)_3\ C_3H_5 + 3H_2O = 3C_{18}H_{34}O_2 + C_3H_8O_3$$

$$C_{18}H_{34}O_2 + 18O_2{}^{*} = C_6H_{10}O_5 + \underset{\searrow}{C_2H_8O_3} + 10CO_2 + 8H_2O$$

$$\frac{10\ CO_2}{18\ O_2} = 0.55\ \text{R.Q. (hypothetical “unknown substance”)}$$

The total R.Q. would depend upon how much fat and carbohydrate were being completely oxidized to CO_2 and H_2O at the same time. These equations would result in the formation of 58.3 gm. of starch from 100 gm. fat. Detmer's analyses for the hemp seed show a loss of 15.56 per cent of fat and the neoformation of 8.64 per cent of starch in its place during the first 7 days of germination of the hemp seed. For 15.56 gm. fat Godlewski's equations would yield 9.07 gm. carbohydrate. This agreement makes it highly probable that the destruction of fat actually runs some such course.

Detmer did not find any soluble carbohydrate in his analyses of germinated hemp seeds, but Frankfurt (13) working with sunflower seeds, which have about the same composition, reported increasing quantities of soluble carbohydrates. Green (14) later identified cane sugar in the germinating castor bean in considerable amount, and Rhine (15) reports an abundance of a "non-reducing" sugar both in the castor bean and in the hemp seed of similar stages.

[*] Godlewski uses $17O_2$ which evidently is an error, since his equation does not balance. Ricinolein has a different formula (see Paper III).

Respiratory quotients as low or lower than those found by Godlewski have been reported for fatty seeds by Gerber (16), Iwanow (17), Harrington (18), and Ermakoff and Iwanoff (19). Gerber argues that if the fats stored in the seed are easily oxidized (highly unsaturated) as in flax seed (linolic acid), the R.Q. of germination will be near that for complete oxidation of fat; but it they are difficultly oxidized (less unsaturated) as in castor bean (ricinoleic acid), the R.Q. will be near that for complete conversion of fat to carbohydrate; *i.e.*, in the neighborhood of 0.3. Sherman (20) has published a table giving respiratory quotients of many seeds both dormant and germinating. Amongst them are several showing that the R.Q. of the endosperm is lower than that of the embryo of the same seed, taken presumably at the same stage of germination. These are for starchy seeds. Sherman gives no similar comparisons for fatty seeds and the present writer has been unable to find any such in the literature. This aspect of the subject is one of considerable significance for the main thesis, as will be shown.

Rhine (15) has developed the fact that the hypocotyl of starchy seeds (pea, wheat, barley, etc.) and that of fatty seeds (cotton, sunflower), taken separately from the rest of the seed in germination, exhibit the same R.Q.; namely, 0.77 on the average. He offers this similarity as proof that fat is transported from the endosperm into the hypocotyl through the cotyledon, not as fat, not even from fatty seeds, but always as sugar, and that any fat found in any part of the young plant is there as the result of synthesis from carbohydrate, as in starchy seeds. "The evidence . . . . favors the view that all the fat stored in the fatty seed is, as we have known most of it to be, first converted to sugars before being transported."

Terroine and associates (21) have shown by bomb calorimetry that the yield of energy to the young plant expressed as percentage of the energy lost in the process of germination (the resulting coefficient is called the energy yield) is greatest in starchy seeds, next in proteinous seeds, and least in fatty seeds, which are also fairly high in protein.

	Average composition			$\frac{\text{Energy of the young plant} \times 100}{\text{Energy expended}}$
	Protein	Carbohydrate	Fat	
Sorghum	9	84	7	73 (Rice and sorghum)
Lentil	31	62	7	62 (Pea and lentil)
Peanut	16	9	75	54 (Flax and peanut)

If protein is substituted for carbohydrate in the lentil, as compared with sorghum, *i.e.* from 9 to 31 per cent, the energy yield is depressed 11 per cent (73 to 62) but replacing carbohydrate with fat, in peanut as compared with lentil, *i.e.* from 7 to 75 per cent, reduces the energy yield 8 per cent more. In a second paper Terroine (22), using considerably different figures for composition and slightly different for his energy yield, makes out that the first substitution just mentioned, amounting to a change in 28.7 per cent of the weight of the seed from carbohydrate to protein, results in a loss of efficiency of 10 per cent, which, for 100 per cent substitution, would give a difference of 34.8 per cent in efficiency. Passing from rice (or sorghum) to peanut there would be a reduced total efficiency of 19 per cent (as above), but 4 per cent of this is due to protein change on the basis just given (except that the percentage of protein in peanut now is 21+ per cent) while 15 per cent is due to a change of 64.4 per cent (table above is 66) in weight from carbohydrate to fat. If a change of 64.4 per cent carbohydrate to fat produces a reduced energy yield of 15 per cent, a complete change, or 100 per cent, would produce a lost efficiency of 23 per cent, which agrees with the computation of Zuntz on the assumption that all the carbon of the fat is found in the carbon of the carbohydrate. Godlewski's equation obviously does not give this result.

Malhotra (23) studied the energy changes of some fatty seeds (castor bean, peanut, flax, and hemp) along with other seeds, during the first 8 days of germination, and in the case of the castor bean recorded a total loss of 0.96 cal. per unit weight due to loss of weight and change in chemical composition. He gives no opinion regarding the nature of this change and reports an R.Q. for the 4th day of germination of 0.71.

Ermakoff and Iwanoff (19) investigated the possibility of partial oxidation of unsaturated fatty acids, according to the conception of Warburg (24), in flax seed, but found no change in the iodine number or in the refractive index of the oil found at different stages of germination. They conclude that the low quotients are due entirely to transformation of the fats to carbohydrate.

EXPERIMENTAL

The choice of the castor bean as representative of the oleaginous seeds was made partly because a single specimen is large enough to give an easily measurable rate of oxygen consumption and partly because it was being used in the laboratory as a source of vegetable lipase. A few experiments, given in Table I, were made with flax seed; but they proved difficult to handle, mainly because of their small size, and, for the same reason, the rate of oxygen consumption was so slow as to require many hours for a single determination with as many as three or four seeds at a time.

The so called Warburg method was adopted. The only new feature introduced by Warburg is the modification in the volumetric bottle of Brodie (25) necessary to adapt it to the study of tissue respiration. It happened that the well inside the bottle introduced by Warburg for containing alkali solution, was, in the volumetric bottles used, of just the right dimensions to support a germinating castor bean on its rim (Fig. 1). Barium hydrate or KOH of N/5 concentration was employed in the bottom as absorbent for CO_2 and N/2 HCl in the "sac" or bay off the volumetric bottle, for discharging the CO_2 after the oxygen measurement had been made. In this way both determinations could be accomplished with the same bottle. The methods of manipulation and calculation are well described, in general, by Richardson (26).

A protocol which, as it happened, gave perfect agreement between the respiratory quotients of two beans, is given below. Such perfection was rare. It was not unusual, however, to obtain agreement in oxygen absorption in successive experiments on the same bean within 3 to 6 per cent. The CO_2 was seldom within this range in successive experiments.

Preparatory to germination the beans were treated with 0.8 per cent formaldehyde for 5 minutes, then rinsed at least four times in sterile distilled water. They were then placed on a glass plate between filter papers which had been soaked in the same formaldehyde solution for 15 minutes and then rinsed thoroughly in hot sterile distilled water. The filter papers were covered by an inverted copper water bath, with side tube for admitting air and the glass plate placed away in a dark closet for germination at room temperature, which, at the time these experiments were carried out (July, August, and early September), was on the average about 25°C. The filter papers were moistened occasionally with sterile distilled water. Whenever mould developed on the beans, as happened occasionally, they were discarded. It required in the neighborhood of 3 days to bring the beans to what will be called in these papers the "first stage" of germination; namely, a length of hypocotyl (radicle) not to exceed 10 mm. Actually, of course, the first stage is the stage of swelling before the radicle appears. 2 days more would permit of growth to a length, quite often, of 20 mm. Beans 19 and 20 given in the protocol had been germinating 6 days.

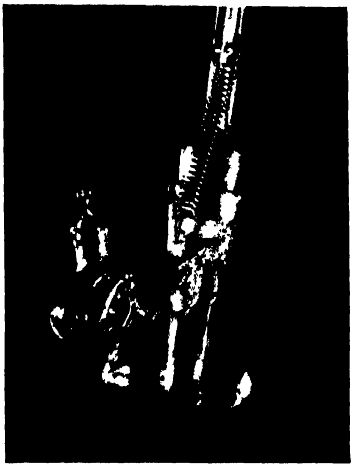

FIG. 1. A castor bean with well developed hypocotyl inside the respirometer bottle resting on top of the well.

The temperature of the bath in which the manometer bottles were immersed was regulated to 30–31°C. in all the experiments on flax seeds and on single beans reported here, and practically always remained constant within 0.1° during the period of observation. The control manometers in any case corrected for both thermal and barometric changes. An additional manometer corrected also for any CO_2 contained as carbonate in the barium hydrate solution.

The manometers were attached to a shaking device.

The "stages" of germination adopted in these studies are as follows:

1st	stage, length of hypocotyl	5- 10 mm.
2nd	" " " "	10-20 "
3rd	" " " "	20- 35 "
4th	" " " "	35- 45 "
5th	" " " "	45- 60 "
6th	" " " "	60- 80 "
7th	" " " "	80-100 "
8th	" " " "	above 100 mm.

(See also Table I of Paper III.)

Protocol

Manometer No	8	9	10	11	12
	cc.	cc.	cc.	cc.	cc.
Volume H_2O (in well)	0.5	0.5	0.5	0.5	0.5
" N/2 HCl (in sac)	1.0	1.0	1.0	1.0	1.0
" N/5 Ba $(OH)_2$ in bottle	1.0	1.0	1.0	1.0	1.0
" of beans		No. 19,0.6	No. 20,0.4		
Volume total		3.1	2.9		
Time of placing bean in bottle, *p.m*		1:32	1:35:30		
Closing stop-cocks *start* (temperature 30.25°), *p.m*	1:40:30	1:42	1:43	1:43:20	1:44
Readings O_2 at start, *mm*	70.0	69.5	70.0	69.0	69.0
While shaking, *end* (temperature 30.25°), *p.m*	2:08:30	2:08:30	2:10	2:11	2:12
Readings O_2 at end, *mm*	80.0	−31.5	−35.0	78.0	78.0
HCl dumped at, *p.m*		2:09	2:10		2:12
While shaking, *final* (temperature 30.25°), *p.m*	2:13	2:13	2:13	2:13:30	2:14
Readings for CO_2 end, *mm*	83.0	+25.0	+28.0	80.5	83.5

Calculation Bean 19, Manometer 9

O_2, +69.5 to −31.5, less change for No. 8 control, +91 mm.

91 × 2.99 (vessel constant) = 272.09 c.mm. O_2 in 26.5 min.

CO_2, −31.5 to +25, less change for No. 12 control = 51.0 mm.
51.0 × 3.15 (vessel constant for CO_2) = 160.6 c.mm. CO_2 in 41 min.
272.09 c.mm. O_2 in 26.5 min. = 420.9 c.mm. in 41 min.
160.6/420.9 = 0.381 R.Q.
Bean 20 184.0/481.2 = 0.382 R.Q.
Bean 19, length of hypocotyl 32 mm.
Bean 20, length of hypocotyl 33 mm.

Irwin (27) has raised the question whether high respiratory quotients obtained from animal organisms under the action of ether may not be due to the splitting of carbonates by the action of organic acid formed in the process of metabolism, and has shown that this is not true of plant tissues. If carbonates were formed and retained in the endosperm during germination of fatty seeds a respiratory quotient below that for combustion of fat might be produced and the inference of conversion to carbohydrate would be false. This possibility was controlled by several experiments of which the following is representative. A crushed germinating bean was introduced into the bottom of a manometer bottle and this manometer equilibrated with a control empty bottle. HCl of the usual strength was then dumped from the sac onto the crushed bean and readings taken immediately and after shaking for 6 minutes. Readings were taken as follows:

Manometer No.	10	11
	Crushed bean	Empty
Readings at 5:22 p.m.	73.8	68.0
HCl dumped 5:23 "		
5:27 "	74.0	70.0 correction − 2 mm.
Manometers shaken 5:33 p.m.	76.0	69.0 " − 1 "

Maximum possible effect due to liberation of CO_2 from carbonates therefore would be 2.2 mm., which at the usual level of readings might raise the quotient as much as 0.03, rarely more. Acid dumped onto a detached hypocotyl 23 mm. long, in one instance produced a rise of 6.5 mm., and when dumped onto a bean with attached hypocotyl of 10 mm. length produced a rise of 4 mm. Apparently, therefore, carbonates are stored, or at least CO_2 may be retained, in significant amount in the new growth. However, since no question of a conversion of fat to carbohydrate in any part of the new plant is involved, and as will be seen presently the R.Q. here is always in the range of carbohydrate combustion, or indeed of conversion back to fat (see p. 295) no error of interpretation is produced so far as the main thesis is concerned. However, extreme care was always taken to prevent the acid touching any part of the bean in the process of dumping, and when it was known to occur the result was discarded.

RESULTS

1. Short-Period Experiments

Table I presents typical experiments with germinating flax seeds. The stage at which the seeds were introduced into the well (because of the gummy substance enveloping these seeds they adhered readily to the inside wall) and the stage which they had reached when final readings were taken, as well as the elapsed time in hours are indicated. Mm. refers to average length of the hypocotyls. When removed from the well the seed coat and gummy substance were removed by gently

TABLE I

Flax Seed Experiments

Warburg Apparatus

Experiment No.	No. seeds	Stage in	Length of experiment	Stage out	Respiratory exchange		R.Q.
					CO_2	O_2	
				mm.	*c.mm./mg.*	*c.mm./mg.*	
1	3	Tip visible	16 hrs.	24	5.215	9.095	0.570
	3	“ “	16 “	27	11.02	14.75	0.750
2	3	1–3 mm.	19 “	12.5	6.65	11.97	0.550
3	2	Tip visible	23 “	9.5	8.194	12.678	0.646
			23 “	15		8.613	0.672
4	2	“ “	9 hrs. 40 min.	22	10.79	14.918	0.765
			“ “	24	10.98	15.13	0.730
5	2	5–8 mm.	8 hrs.	20	5.72	11.27	0.510
6	4	Tip visible	6.5 “	19.9	5.09	7.41	0.606
	4	“ “	6.5 “	23	5.09	7.79	0.653
7	4	“ “	7.5 “	20	7.81	12.23	0.640
					Total		
8	3	5 mm.	7.75 “	22	159	241	0.659
	3	“	7.75 “	21	133	273	0.487
Average							0.633

pressing out the naked endosperm and the total moist weight was obtained immediately. The CO_2 and O_2 are expressed as c.mm. per mg. of this moist weight. Some of the rather large variations shown are probably due to errors in securing the moist weight, others to injury to the very delicate endosperm and hypocotyl. Dry weights would have been preferable. However, these experiments were purely preliminary and were not followed up for reasons already given. They are shown merely as confirmatory of the low quotients obtainable with a seed which contains only around 37 per cent (Winton) fat and 27 to 30 per cent carbohydrate. In all but two instances the R.Q. is well below the level of fat combustion. Since carbohydrate combustion is not excluded the quotients are doubtless significant of some other use of oxygen than combustion. Gerber's idea regarding the effect of highly unsaturated fatty acids (p. 285) should also be borne in mind.

Table II presents typical experiments with single, entire, germinating castor beans. Because of their large size and very active metabolism, as compared with the flax seeds, these experiments could be completed often in as many minutes as the others required hours. It is difficult to obtain accurate dry weights on seeds containing as much fat as this bean without extraction with fat solvents. The results in this table, therefore, are expressed without reduction to any unit of weight. That it is possible to obtain fair agreement between different beans of approximately the same size and stage of development when expressed in terms of moist or dry weight is seen from Table III.

The respiratory quotients agree rather better than the rates of metabolism. This is true also of successive experiments performed on the same bean on the same day, as may be seen from Table II, particularly for Beans 12 and 16. Other experiments not shown in any of the tables bear out this conclusion, which, otherwise stated, would indicate that the nature of the chemical processes involved is more constant for any given stage than the rate of change.

From Table II it is quite evident that the chemical process responsible for the low quotient proceeds at different rates at different stages of the germination. To give the exact quotient, characteristic of each stage has not been undertaken in this study, for it is not possible to confirm any conclusion reached through respiration experiments on single beans by chemical analyses. That will be undertaken in the third paper of this series.

TABLE II

Castor Bean Experiments

Single Bean in Warburg Apparatus

Variation with Stage

Bean No.	Stage of growth. Length of hypocotyl	Length of experiment	Respiratory exchange		R.Q.
			CO_2	O_2	
	mm.	*min.*	*c.mm., total*	*c.mm., total*	
13	12	69.0	234.9	345.6	0.679
14	12+	46.5	155.8	231.4	0.535
17 (1)	16	30.0	208.2	337.8	0.617
17 (2)	16	32.0	211.4	407.8	0.518
16 (1)	19	32.0	142.6	247.6	0.576
16 (2)	19	32.0	130.0	223.3	0.582
Average					0.585
18	20	15.0	85.4	252.9	0.337
12 (1)	23	34.5	117.3	406.3	0.289
12 (2)	23	23.0	107.8	287.4	0.373
12 (3)	23	22.5	98.0	320.4	0.306
10	24	31.5	146.7	413.8	0.355
11	29	32.5	127.9	496.9	0.257
19	32	41.0	160.6	420.9	0.382
20	33	37.5	184.0	481.2	0.382
Average 20 to 30 mm.					0.323

TABLE III

Respiratory Metabolism of Castor Beans of Similar Stages of Development Per Unit of Weight

Bean No.	Moist weight	Dry weight	CO_2	O_2	R.Q.
	gm.	*mg.*	*c.mm./100 mg. (dry)/min.*	*c.mm./100 mg. (dry)/min.*	
42	0.684	305	1.92	4.9	0.39
43	0.520	262	0.84	3.5	0.24
44	0.449	229	2.13	6.2	0.34
45	0.453	232	2.36	5.6	0.42

The next phase of the subject of special interest is the relation of the respiratory exchange of the embryo to that of the endosperm. If fat were changed to sugar in the endosperm and after diffusion through the cotyledon into the hypocotyl were there either oxidized as such or converted back to fat, the R.Q. of the hypocotyl would necessarily be considerably higher than that of the endosperm. As noted on page 285 such a difference has been demonstrated for starchy seeds. It should *a fortiori* be true of fatty seeds. Fortunately it was found to be very easy to strip the cotyledons out of the endosperm and to determine their metabolism in the same apparatus as the entire germinating bean, and immediately thereafter. Typical determinations of this kind are given in Table IV. In each case shown the R.Q. of the germinating bean as a whole was determined, and, as soon as this was finished, the bottles were cleaned and prepared for another observation, the bean meantime being kept in moist filter paper. The cotyledons then were carefully separated from the endosperm and with the hypocotyl attached were placed immediately in the identical bottles in which the parent beans had been observed. Naturally it required a considerably longer time to obtain figures of the same order of magnitude for the two gases from the young plant, because of its much lighter weight. Table IV shows that this time was from three to four times as long.

TABLE IV

Respiratory Exchange of Whole Germinating Bean Compared with That of Young Plant

No.	Part	Stage	Length of experiment	CO_2	O_2	R.Q.
			min.	*c.mm.*	*c.mm.*	
26	Whole bean	3 branches	16	149.3	490.5	0.304
26	Young plant	3 "	77	305.2	348.8	0.974
27	Whole bean	12+ "	19	131.1	386.0	0.339
27	Young plant	12+ "	72	311.3	315.6	0.986
28	Whole bean	60 mm. 3 branches	23	189.4	500.8	0.378
28	Young plant	60 " 3 "	68	279.8	324.2	0.863
29	Whole bean	30 " 3 "	21	142.9	269.7	0.328
29	Young plant	30 " 3 "	65	260.0	280.0	0.928

In every case shown, and in several other determinations not shown, the production of carbon dioxide was more than twice as great in the young plant as in the endosperm, in proportion to oxygen used up. The result was a quotient for the young plant well up in the range of carbohydrate combustion, while that of the entire germinating bean was, as usual for the stage selected, within the range for complete conversion of fat to carbohydrate. Sketches of the cotyledons and hypocotyls of these four beans, in natural size, are shown in Fig. 2. The results are strongly indicative of the sequence of chemical changes postulated by Detmer, Godlewski, Iwanow, Rhine, and Ermakoff and Iwanoff. However, physiologists are all too prone to accept an explanation which appears plausible, as already proved, because a *possible chemical reaction* can be written for such a change. Proof in any adequate sense of the word is not given until the *actual chemical changes are shown to occur* at the same time, as the respiratory signs. A number of other possibilities exist. For example, Green (14) has shown that an organic acid which he was not able to identify is formed

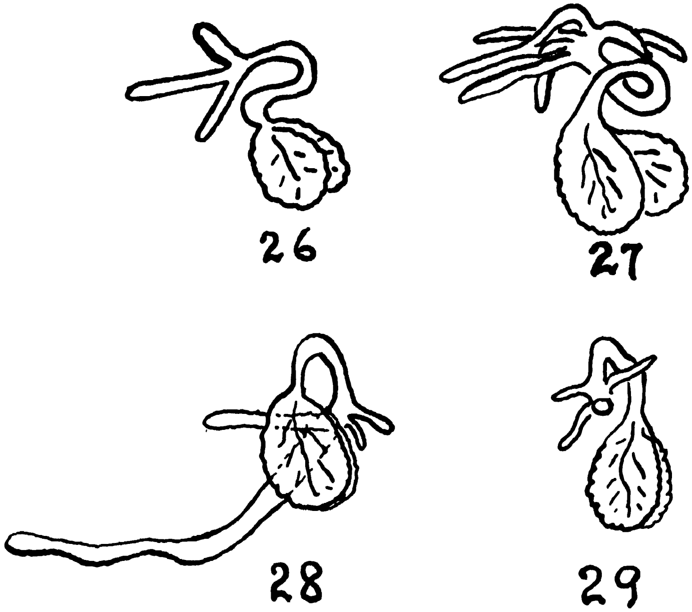

FIG. 2. Cotyledons and hypocotyl (entire young plant) of Beans 26, 27, 28, and 29, metabolism of which is shown in Table IV.

in the germination of the castor bean. If this were formed *de novo*, *i.e.* not merely by hydrolysis of fat, as is suggested by Godlewski's equation, its production would require extra oxygen just as does the formation of starch or sugar from fat. This aspect of the problem will be gone into in the third paper. It is sufficient for the moment to conclude that the respiratory exchanges are consistent in general with the chemical changes postulated.

Two further points should be mentioned, before we leave this division of the subject. One, that the respiratory quotients of the entire germinating bean almost prove too much. They are so low—so much lower than the theoretical for Godlewski's equation (p. 284)—that they quite exclude that reaction as the only possible one. When we consider that the metabolism of the young plant with its high quotient is included at least in part in these low ones and also that the endosperm itself doubtless is oxidizing some fat or carbohydrate, the wonder is that quotients of the order obtained are possible. Nevertheless it is believed they are correct. Certainly they are consistent and not at all exceptional for these stages. Some further light would be shed on their significance, if it were possible to take the metabolism of the endosperm also separately. This has been done several times and typical results are found in Table V. Two circumstances, however, detract from the value of these experiments for the purpose intended. It is impossible to separate the cotyledons from the endosperm without removing the seed coat. Other experiments, not shown

TABLE V

Respiratory Exchange of Whole Bean, Endosperm, and New Plant

No.	Part	Stage	Length of experiment	CO_2	O_2	R.Q.
			min.	*c.mm.*	*c.mm.*	
32	Whole bean	40 mm. 1 branch	59.5	236.2	612.2	0.386
32	Endosperm	40 " 1 "	52.0	195.9	426.8	0.459
32	New plant	40 " 1 "	72.0	155.8	140.8	1.106
33	Whole bean	45 mm. 4 branches	57.0	292.9	604.5	0.484
33	Endosperm	45 " 4 "	44.5	184.0	331.7	0.555
33	New plant	45 " 4 "	70.0	173.3	192.5	0.90

in the tables, proved that when a germinating whole bean was observed with the seed coat lacking, the respiratory quotient was a little higher for the first 45 minutes than when the coat was in place. The difference was great enough to vitiate the quotients in Table V somewhat. The quotient of the endosperm alone, it was expected, should be lower than that of the germinating whole bean, since the R.Q. of the young plant alone is so much higher. That Table V does not exhibit this relationship is due, certainly to some extent to the fact that the seed coat had to be removed, and, unfortunately, one cannot say to just what extent this circumstance affects the result. Secondly, the observation with the endosperm and that with the cotyledon and hypocotyl (new plant) did not continue long enough to give comparable total figures with the whole bean experiment. The reason is a good one; namely, to avoid postponement of the observation with the separated parts so long as to permit the metabolism to run down; nevertheless it is regrettable. This circumstance makes the quotients a little less reliable both for the endosperm and the young plant. Possibly the very high R.Q. for young Plant 32 is to be explained in this way.

Having just mentioned the possibility that the metabolism of the isolated parts runs down when separated from their natural relationships, some facts in support should be mentioned briefly. Many times successive experiments on the same whole bean have been carried out the same day. Usually, they have agreed quite satisfactorily. With the young plant the agreement is never so good as with the whole seedling. This would be expected, for the plant is not yet old enough to maintain itself even in its natural habitat, much less in a respirometer bottle. The following illustration will suffice.

Young plant	CO_2 in 100 min.	O_2 in 100 min.	R.Q.
	c.mm.	*c.mm.*	
No. 1, 1st experiment	275	287	0.96
2nd "	222	281	0.79
No. 2, 1st "	227	237	0.96
2nd "	217	246	0.88

The first experiment in each case lasted 100 minutes, the second 51 minutes; they are calculated, however, to the same basis. In each case the R.Q. is significantly lower in the second experiment.

The second point remaining to be discussed briefly is the relationship of the respiratory exchanges on the basis of unit weight. Several of these experiments also were carried out and two are shown in Table VI. Moist weights were used. The whole germinating bean was weighed minus its seed coat just after the metabolism observation. The endosperm and young plant were weighed immediately after separating them and the endosperm was kept moist until its metabolism could be determined. Thus the three weights were obtained under strictly comparable conditions, and since the fat content of the endosperm is so very much higher than that of the young plant, it is believed the moist basis is just as satisfactory as the dry.

TABLE VI

Whole Seedling, Endosperm, and New Plant Calculated to Unit Time and Weight

No.	Part	Moist weight	CO_2	O_2	R.Q.
		mg.	*c.mm./hr./100 mg.*	*c.mm./hr./100 mg.*	
36	Whole seedling	911	52.6	133.4	0.394
36	Endosperm	575	53.0	140.9	0.378
36	Young plant	144	139.0	180.0	0.776
37	Whole seedling	1047	47.6	104.6	0.454
37	Endosperm	601	53.2	111.1	0.468
37	Young plant	194	141.5	170.5	0.829

It was not surprising to find the metabolism of the young plant much higher than that of the entire bean. In one of the cases (No. 36) shown it is approximately 35 per cent higher on the basis of the oxygen consumption and in the other about 63 per cent. In each case the metabolism of the endosperm is 5 to 7 per cent higher than that of the whole germinating structure, likewise on the basis of the oxygen. This again indicates that the metabolism of the whole bean is somewhat held in check by the seed coat, for the endosperm necessarily was observed devoid of the coat.

Still more interesting are the CO_2 relationships. The endosperm and whole bean give off approximately the same volume per unit of weight, but the young plant gives off 2.6 times as much as either whole bean or endosperm. At first sight this seems like a clear case of temporary storage of CO_2 and its escape from the cotyledon as soon as it is withdrawn from its housing in the interior of the seed. Experiments have already been referred to (p. 289) which prove that this cannot be true of the hypocotyl. The same sort of experiments were done with the cotyledon. No readings have been preserved, unfortunately, but acid applied to the cotyledon causes the evolution of very little gas.[1] There is no question, therefore, that the large output of CO_2 from the young plant is due to active metabolism. Can any special significance be attached to this fact?

If the statement just made is turned the other way about, it is clear that on the basis of unit weight, the peculiar metabolism which goes on in the endosperm to give very low quotients differs from that of the young plant much more on the basis of CO_2 than on the basis of oxygen. One might have expected that when reduced to unit weight the endosperm would show a greater absorption of oxygen than the young plant, the CO_2 remaining more nearly the same. The statement usually made is that originally reported by de Saussure—the oxygen absorption is much greater than the CO_2 elimination. The indication from these few experiments is rather that CO_2 is produced but held back to form carbohydrate and when the carbohydrate later is broken down the CO_2 is liberated. If the carbohydrate were to be transformed to fat, it would be accomplished by elimination of still more CO_2. Fat is found in the protoplasm of the hypocotyl (Rhine (15)); hence a part of the surplus CO_2 coming from the young plant may be from this reaction. It will require more experiments of the same kind and others of special design to make sure of this as a fact. At present it may be remarked only that Chauveau's (28) conception is favored rather than that of Bleibtreu (29) concerning the nature of the chemical change necessary to produce fat from carbohydrate and *vice versa*. (See, however, the evidence in Paper II.)

2. *Long-Period Experiments*

The short-period experiments are interesting from many points of view; but with single beans the physiological results cannot be checked by chemical analyses of the same material. A second type of experiment was designed for this purpose. The chemical analyses will not be reported in this paper but in Paper III. A description of the respiration method may be given at this time, however, and a few typical results merely to confirm for long periods the character of the respiratory quotients reported above for the entire germinating bean in single cases.

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[1] Later check experiments done at our request by Dr. M. Elizabeth Marsh, showed that a maximum of 10–15 c.mm. of gas could be obtained from a single pair of cotyledons. This would lower the R.Q. of the young plant in Tables V and VI about 0.05 if all the CO_2 stored should have been given off in the respiration period, which is very unlikely.

Beans were started germinating in the usual way (p. 287) and when they had arrived at some definite stage, *e.g.* our first stage where they had broken the seed coat and put out a little radicle anywhere from 1 to 5 mm. long, a number of these were selected from their healthy appearance and placed in an air-tight bottle of 1 to 4 liters capacity which had been sterilized in a suitable manner. The bottles were provided with a rubber stopper containing two glass tubes closed by means of tight-fitting rubber tubes and screw clamps. The beans were placed on sterile filter paper moistened with freshly sterilized distilled water and the bottles ventilated with filtered outside air. The bottles were then tightly closed and were covered with black paper or tin foil. They were placed in dark cupboards for several days. When by inspection it was found that the beans had made a satisfactory growth, samples of air were drawn from one tubulure; while admitting water or mercury through the other. The samples were analyzed by means of a special Haldane apparatus designed as to the burette and some other features by Dr. Nasset (30).

Table VII shows the results of six such experiments. The first three (Nos. 2, 3, and 5) were first stage, the next two (Nos. 4 and 6) were second stage, and the last enumerated (No. 1) was third stage, when confined. The fourth column gives the number of days of confinement and the fifth the stage of growth reached by the beans when the air was sampled. The last columns give the respiratory exchange in terms of percentage alteration in the composition of the air. Some of these figures suggest, at first sight, that more oxygen was removed than could have been present in the air at the start (20.93 per cent). However, this will not be misleading to anybody familiar with air analysis, for the percentage change is found by subtracting the percentage formed at the end, not from 20.93 but from a figure proportional to the nitrogen remaining at the end. In these cases (Experiments 1, 2, 3, and 6) the oxygen was practically all used up. In these also the CO_2 percentages found at the end were excessively high. It is not certain that the beans were alive in every case. Indeed in No. 2 the odor of putrefaction was unmistakable, and this condition doubtless is responsible for the exceptional respiratory quotient found. The other quotients are consistent in indicating the formation of fat from carbohydrate.

In such experiments where the amount of material involved is sufficiently large to permit of exact chemical analysis, it will be possible to check the respiratory findings by actual composition at the beginning and at the end. Results of this character will be found in Paper III.

TABLE VII

Bean-Bottle Experiments

Experiment No.	No. of beans	Stage in. Average length hypocotyl	Days in	Stage out. Average length hypocotyl	CO_2 gain	O_2 loss	R.Q.
		mm.		*mm.*	*per cent*	*per cent*	
5	24	3	3	13.1	4.63	8.73	0.530
					4.70	8.64	0.544
2	6	5	8	50	15.96	22.15	0.72*
3	6	5	5	35	10.36	21.98	0.471
					10.63	21.96	0.484
6	24	15.6	3	29.2	10.65	21.05	0.506
					10.90	20.86	0.569
4	6	17.5	2	20	9.52	18.59	0.514
					9.13	18.33	0.498
1	5	38	8	89	11.62	21.68	0.538

* Spoiled—putrefaction.

SUMMARY AND CONCLUSIONS

1. Respiration studies on single castor beans, made by means of the Brodie-Warburg method, at various times after the start of germination, as well as studies on groups of germinating beans over periods of 3 to 8 days, made by a simple procedure involving analysis of the respired air by the Haldane method, consistently give respiratory quotients from 0.30 to 0.58, indicating the conversion of the oil to carbohydrate.

2. The R.Q. varies with the stage of germination, the lowest point occurring when the new growth (hypocotyl) measures from 20 to 35 mm. in length.

3. The R.Q. of the young plant (cotyledons and hypocotyl), separated from the endosperm and studied in the same apparatus, varies from 0.78 to 1.00. It is invariably high enough to indicate considerable combustion of sugar. The R.Q. of the endosperm alone is low, but usually somewhat higher than that of the entire germinating structure.

4. On the same unit of moist weight the young plant (cotyledons and hypocotyl) produces about 2.6 times as much CO_2 as the endosperm, whereas it absorbs only 1.3 times as much O_2.

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THE CONVERSION OF FAT TO CARBOHYDRATE IN THE GERMINATING CASTOR BEAN

II. THE COMBUSTION RESPIRATORY QUOTIENT AS DETERMINED BY A MODIFIED OXYCALORIMETER

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The castor bean belongs to that group of seeds that contain large quantities of fat and no carbohydrate before germination. As germination proceeds the fat stores decrease and carbohydrate appears (Green, 1890) (see also Paper III following). The purpose of this investigation was to determine whether or not the combustion respiratory quotient rose with succeeding stages of germination, thus indicating a change from an oxygen-poor to an oxygen-rich substance.

Apparatus

By combining the Benedict and Fox (1925) oxycalorimeter and the Benedict universal metabolism apparatus and introducing a more effective cooler, one is enabled to obtain accurate respiratory quotients upon the combustion of small amounts of thoroughly dried plant or animal material. Fig. 1 is a diagrammatic illustration of the arrangement. The cooler is made from a hot water heater coil installed in a large metal can filled with cold water. The water bottle preceding the spirometer is inserted in order partially to control the temperature of the air. If one is very careful to keep drafts from the spirometer bell and quickly cool the Pyrex lamp chimney with a cold wet cloth immediately following the combustion, there is no difficulty in keeping the temperature of the air in the spirometer at the same point as it was before combustion. This eliminates temperature change corrections.

In order to check the accuracy of the apparatus C.P. sucrose was used. Checks with sugar were also made at various intervals during the experiments on the castor beans. Table I is representative of the values obtained for sucrose.

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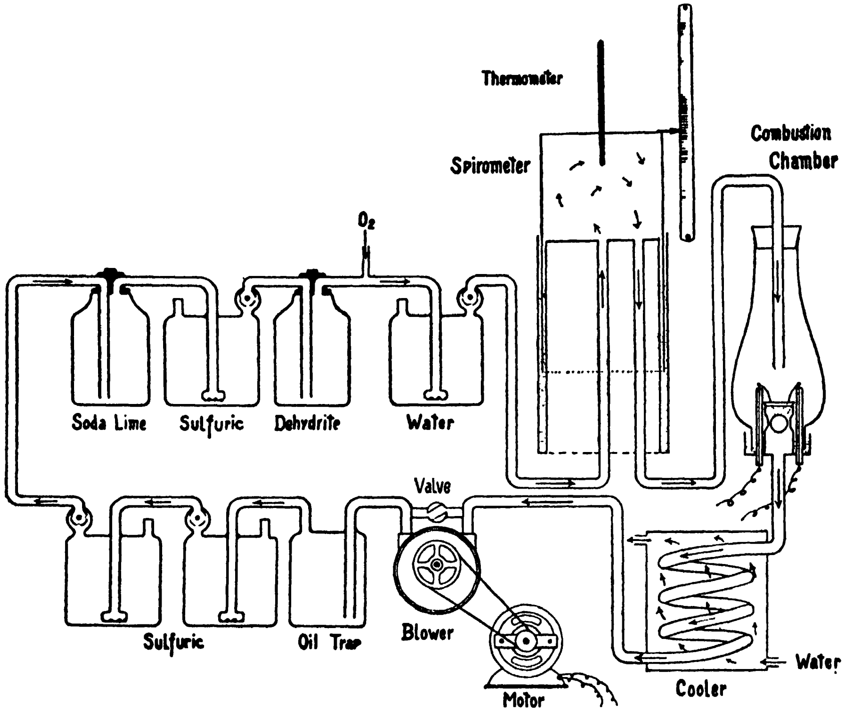

FIG. 1

TABLE I

O_2	CO_2	R.Q.
liters per gm.	*liters per gm.*	
0.7838	0.7850	1.001
0.7790	0.7730	0.992
0.7677	0.7731	1.007
0.7558	0.7560	1.000
0.7844	0.7817	0.996
0.7675	0.7794	1.015
0.7602	0.7606	1.000
Average....0.7712	0.7727	1.002

EXPERIMENTAL

The pericarp of the ungerminated bean was removed and the remainder of the bean immediately weighed and placed in the combustion chamber. Sufficient oil is present to insure good combustion without drying. The results obtained by burning the ungerminated bean in the moist state are shown in Table II. Generally two whole beans were used for each combustion.

Good sound hard beans were selected and placed in a filtered solution made from 10 gm. of commercial chloride of lime and 140 cc. of water (Wilson, 1915). They were kept in this chlorine solution for 15 minutes in order to sterilize them against bacteria and mold. After this treatment they were individually washed in sterile water to remove the excess chlorine and placed between layers of damp sterile filter paper on a piece of window glass. A sterile water bath was inverted over them excluding all light but permitting a small amount of ventilation through an opening loosely filled with cotton and shielded from the light. As required, the filter paper was dampened with sterile water. When the desired stage of germination was reached, the endosperm was freed from the clinging pericarp and the entire bean and young plant cut into small pieces and placed in the vacuum desiccator until thoroughly dehydrated. The stages of germination were chosen arbitrarily depending only upon the length of the hypocotyl.

TABLE II

Length of hypocotyl	O_2	CO_2	R.Q.	No. of experiments on which average is based
mm.	*liters per gm.*	*liters per gm.*		
Ungerminated	1.542	1.166	0.756	12
Average 5.0	1.550	1.169	0.754	10
" 16.3	1.527	1.168	0.765	9
" 33.6	1.359	1.110	0.817	8
" 65.0	1.167	1.014	0.869	4
" 112.0	0.992	0.909	0.919	6

There is undoubtedly a greater variation among germinated beans of the same length of new growth than among the ungerminated beans. However, this seemed to be the best criterion since the number of days of germination and other means of determining different stages of germination showed greater variations. Table II gives a summary of the findings. It is rather unsatisfactory to try to germinate the seeds in the dark by the method described (see Paper III for a more successful method), much farther than to a length of hypocotyl of slightly over 100 mm., because the tip of the radicle becomes quite darkened and begins to die. However, a few beans were successfully germinated to a greater length as can be seen in the graph (Fig. 2), in which the data are summarized. It is clear that as germination proceeds the plant gains in an oxygen-rich substance and becomes poorer in an oxygen-poor substance, for the volume of

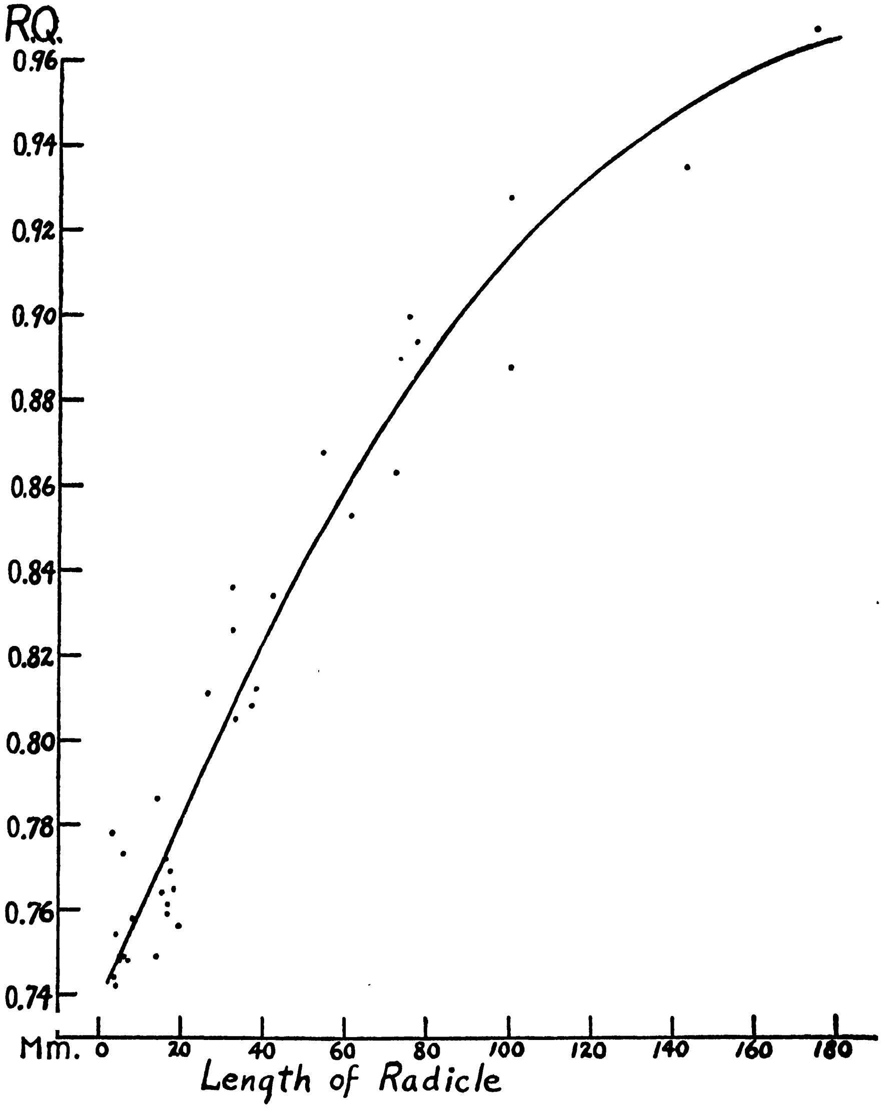

FIG. 2

oxygen required for combustion of the entire germinating seedling grows steadily smaller per gram of dry matter. The volume of CO_2 produced per gram of dry matter, however, remains nearly the same.

There is therefore more oxygen already present per unit of carbon contained in the dry matter.

To check again the efficiency of the apparatus used to obtain the above data, organic combustions were done on a few of the beans. An ordinary gas combustion furnace was used. The carbon dioxide was collected in potassium hydroxide and the water in sulfuric acid. The ash and the nitrogen were determined and the oxygen contained in the bean obtained by difference. Knowing these factors one can easily calculate the additional oxygen used in the combustion and thus determine a respiratory quotient. Table III gives the data obtained by organic combustion. The results check fairly well with those obtained with the modified oxycalorimeter.

TABLE III

Length of hypocotyl	O_2	CO_2	R.Q.
mm.	*liters per gm.*	*liters per gm.*	
Ungerminated	1.548	1.158	0.748
"	1.530	1.147	0.750
"	1.501	1.131	0.754
17	1.571	1.180	0.751
150	0.897	0.824	0.920

In an attempt to find where the oxygen-rich substance was most abundant in the germinated bean, several combustions were done on the young plant alone (cotyledons, hypocotyl, and rootlets) and on the remaining endosperm. Table IV is representative of the results obtained.

It will be noticed that there is no consistent change in the R. Q. of the young plant with respect to the stage of germination, thus indicating that the new tissue formed remains of substantially the same composition. The R. Q. of the endosperm, however, increases with the stage of germination. This doubtless means that the seat of formation of the oxygen-rich substance is in the endosperm. It is of interest to check the weighted respiratory quotients obtained from Table IV with the combustion respiratory quotients found in Table II. The endosperms of the 35 mm. group in Table IV weighed (dry) 2.1356 gm. giving an R.Q. of 0.813 and the young plants weighed 0.2087 gm. with an R.Q. of 0.932. The resultant R.Q. of the combination would be 0.823 which checks fairly well with an R.Q. of 0.817 for a group of 33.6 mm. length shown in Table II. Taking another example, the endosperms of the 113 mm. group in Table IV weighed (dry) 1.7505 gm. with an R.Q. of 0.859 and the young plant 1.0298 gm. with an R.Q. of 0.966. On calculating the proportional amounts of each the resultant R.Q. is 0.899. The R.Q. for the 112 mm. group in Table II is 0.919.

TABLE IV

Average length of hypocotyl *mm.*	O_2	CO_2	R.Q.
	Endosperm		
	liters per gm.	*liters per gm.*	
35	1.452	1.181	0.813
52	1.378	1.129	0.819
85	1.171	0.988	0.843
95	1.211	1.034	0.854
113	1.163	0.999	0.859
134	1.306	1.133	0.868
	Young plant		
35	1.230	1.146	0.932
52	1.074	1.033	0.962
85	0.903	0.847	0.938
95	0.934	0.884	0.947
113	0.908	0.877	0.966
134	1.014	0.940	0.927

The exact nature of the material formed to produce the change in combustion R.Q. is not revealed by this study. Taken in conjunction with the results given in Paper I and the literature cited therein, the observations here recorded are entirely consistent with the view that the fat (oil) of the castor bean is converted to carbohydrate (sugar) in the endosperm in the course of germination, and is at least partly stored in the young plant. The chemical analyses and their correlation with the respiratory quotients will be found in Paper III, immediately following.

SUMMARY

The combustion respiratory quotients of castor beans germinated to various stages, depending upon the length of the hypocotyl, were determined by means of a modified oxycalorimeter.

After germination was well started, the respiratory quotient of the combusted germinated seed increased as the stage of germination increased, indicating a change from an oxygen-poor to an oxygen-rich substance, probably fat to sugar.

The accuracy of the method was checked by organic combustions.

The seat of formation of the oxygen-rich substance is in the endosperm.

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THE CONVERSION OF FAT TO CARBOHYDRATE IN THE GERMINATING CASTOR BEAN

III. THE CHEMICAL ANALYSIS, AND CORRELATION WITH RESPIRATORY EXCHANGE

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In considering the conversion of fat to carbohydrate in the germinating castor bean, two phases of the problem have been discussed in the preceding papers. The results of chemical analyses made on the beans at various stages of their germination will be presented here. The changes in the fat stores of seeds during germination have been studied by numerous investigators who used many different seeds. Hellriegel (1), Leclerc du Sablon (2), Green (3), Green and Jackson (4), Maquenne (5), Deleano (6), Miller (7–8), Mazé (9) and many others have investigated the transformation of oil into starch or sugar during germination. For a more detailed bibliography covering this phase of plant chemistry, the reader is referred to the book by Miller (10) and the articles by Deleano (6) and von Fürth (11). All of the above investigators observed that sugar was formed in increasing amounts as the oil disappeared in germinating fatty seeds, and several noted that the sugar increased up to a certain point and then decreased along with the oil.

The increase in the carbohydrate content in the seedlings of oily seeds necessarily raises the question as to its orgin. Miller (7) working with sunflower seeds found that changes in the protein reserve could not account for the carbohydrate formed. We have observed that there is a slight increase in amino nitrogen in castor beans during their germination, but this increase is exceedingly small, less than 1 per cent. As there is an exceptionally small amount of preformed sugar in ungerminated castor beans it seems likely that the oil must serve as the source of carbohydrate. Miller (10) notes that no one has found free glycerol in germinating seeds or seedlings. However, even if all of the glycerol from the fat which disappeared were utilized for building of carbohydrate, it would not be sufficient to account for that formed. A simple calculation will be presented below in proof of this. Thus it seems evident that the fatty acid fraction must be utilized in carbohydrate production. This statement is substantiated by the investigations of Leclerc du Sablon (12), Maquenne (5), and Ivanow (13) but von Fürth (11) does not agree with their conclusions, or those of Green (3) whose work will be mentioned later. Von Fürth worked with seedlings with a root length (hypocotyl?) of about 40 mm. We will show later that marked changes in the oil and sugar content did not take place until the hypocotyls were over 35–40 mm. in length. Furthermore von Fürth (11) felt that Müntz (14) had no right to conclude that the fatty acids of germinating seeds were converted into oxy-acids since the acetyl value of the oil extracted by von Fürth did not rise during germination. Miller (8) also found no change in the acetyl value of the oil obtained on extraction of germinated seeds. Green (3) isolated a crystalline acid of unknown structure from germinating castor seeds and believed that this might be an intermediary product in the conversion of the oil to sugar. Von Fürth (11) was unable to confirm Green's work, and expressed the belief that if volatile acids, alcohol, acetone, or aldehydes were intermediates between fat and carbohydrate, the amount present at any given time might well be so small as to be undetectable. Deleano (6) realized that many different organic acids could be formed during germination, but he was able to isolate only two, lactic and acetic. Recently Pirschle (15) reported having found considerable amounts of acetaldehyde in germinating fatty seeds, and he believed that this might be the possible intermediate in the process under discussion.

The question as to whether fat is converted directly to starch, glucose, or dextrins (16, 17) or whether numerous substances are formed as intermediates, cannot be discussed profitably at present.

EXPERIMENTAL

Sound castor beans (Brazil seeds) of different sizes and colors were germinated in various ways to determine the best means of procuring successful germination without mould. The most successful method proved to be the following: The selected beans were dipped momentarily into a 1:1000 mercuric chloride solution to destroy mould spores and then introduced into sterilized moist chambers, being placed upon filter paper over sterile cotton, and on top of them was laid another layer of filter paper and cotton. The dish and cotton were sterilized in an oven at 100°C. for 24 hours. The filter papers were moistened with sterile distilled water and the dishes set away in a dark place at room temperature. Water was added as required. Success in preventing mould depends not so much on securing perfect sterility as on prevention of too much moisture in contact with the beans.

The rate of growth not being entirely uniform, the time required for the hypocotyl to reach the length desired was variable. A length of 100 mm. was attained on the average in about 2 weeks, and usually no side branches appeared until the hypocotyl was approximately 35 mm. long. The length of the hypocotyl was selected as a measure of the degree of development rather than the days of germination, in accordance with the experience of Leclerc du Sablon (2), and Deleano (6).

The seedlings were removed from the moist chambers when the hypocotyls reached the desired lengths, the seed coat and sheath pulled off, and the germinating structure dried for about 24 hours at 90°C. Some investigators (6, 18) have recommended drying for a longer time at lower temperatures to prevent changes in the composition of the oil, but soluble carbohydrates might be lost by this slow drying due to respiration of the seed or fermentation brought about by moulds and bacteria. Von Fürth (11) too, has shown that the iodine number of the oil extracted from seedlings dried at 90° for some time has approximately the same value as that of oil extracted from ungerminated seeds. When the plant material was practically dry it was prepared for analysis by grinding into a fine paste or powder, depending upon the amount of oil present.

In a second series of experiments castor beans were dipped in 0.8 to 2 per cent formaldehyde, rinsed in sterile distilled water, and allowed to germinate in the dark. When the hypocotyls were a few millimeters long (see Nos. 7 and 8, Table IV) these seedlings were placed on moist cotton at the bottom of 3 to 8 liter bottles and these were then closed tightly to prevent the loss or entrance of air. The rubber stopper was provided with two glass tubes closed externally by means of rubber tubing and screw clamps, also with a small manometer filled with mercury for recording pressure change in the bottle. The bottles and seedlings were set away in a dark cupboard and after a period of time—the number of days varied—the bottles were removed, a portion of the contained gas being transferred to sampling tubes and analyzed in a Haldane gas analyzer. Ungerminated seeds at times were confined in the same manner. The chief difficulty in these experiments lay in the fact that not all of the seeds germinated or continued to grow. After the gas samples were taken, all the beans or seedlings were removed, dried, ground, and analyzed as set forth in the following paragraphs, analyses being made for water, ash, ether extract, protein, crude fiber, total reducing matter as invert sugar, and in the later experiments glucose.

Moisture was determined by placing samples in shallow evaporating dishes and drying to constant weight at 100°C. These same samples were then ashed in a muffle at dull red heat until the ash assumed a grayish white cast. For ether extract samples of the dried material were weighed by difference into double thickness extraction thimbles and extracted with dry ethyl ether in Soxhlet extractors for 16 hours. In several analyses, samples were extracted with petrolic ether, and the percentage of fat obtained corresponded almost exactly with that obtained with ethyl ether. In one analysis the sample was extracted with both ethyl and petrolic ethers and in the ether extract nitrogen was determined. This amounted to approximately 0.1 per cent, showing that very little nitrogenous matter was removed during two 16 hour extraction periods.

Nitrogen was determined by the Kjeldahl method, the factor 6.25 being used to convert nitrogen to protein. To determine reducing matter a weighed sample of the material was boiled with water for 30 minutes and filtered into a 250 cc. volumetric flask, the residue being washed with hot water. The contents of the flask were cooled and made to 250 cc. 50 cc. portions were removed and put in each of two beakers. To the contents of one beaker, 1 cc. of concentrated HCl was added and the solution heated on a water bath for 15 minutes to hydrolyze the sugar. This was then neutralized with strong NaOH and a drop of HCl added to discharge the pink color of phenolphthalein. The total reducing matter was then determined by the Bertrand method. The reducing substances in the unhydrolyzed 50 cc. portion were determined directly by Bertrand's method. The cubic centimeters of $KMnO_4$ used here were subtracted from the cubic centimeters used for the total reducing matter and the difference calculated as glucose. Zinc sulfate added to the original solution or to the solution after hydrolysis had no effect upon the final results. Repeated boiling of the fat-rich samples failed to yield any additional quantity of sugar. Crude fiber was determined according to the method of the Association of Official Agricultural Chemists (19).

DISCUSSION OF RESULTS

The results of the chemical analysis of beans having hypocotyls from 5 to 100 mm. are presented in Table I. All values are averages of several analyses of beans which had been germinated at different times during the year. The percentage of fat shows a steady decline, the most rapid fall occurring during the period when the hypocotyls are between 35 and 45 mm. in length. Deleano (6) also observed the fact that chemical changes were at a maximum during this stage of germination. As the fat decreases, we notice a marked rise in the reducing matter calculated as invert sugar. There is very little reducing matter in the unhydrolyzed water extracts until the length of the hypocotyl is 35 mm. or more. The crude fiber shows a rather irregular series of values, but there is a definite tendency toward a rise as one would expect. Certainly the accuracy of the method is not of the highest order. Some of the variations may have been due to irregularity in the development of the root system, for some lots of seedlings had far more roots than others. The nitrogen content of the germinated beans was fairly constant, although there is a noticeable rise as germination progresses. This apparent increase in nitrogen content is set forth more clearly in the results shown in Table II. The amino nitrogen content is small, usually less than 1 per cent until the later stages of germination are reached. Loss of a volatile substance during drying would explain this rise in the amount of nitrogen present. The percentage of ash in the seedlings at various times during their growth is fairly constant. There is a gradual increase in the undetermined matter the longer the period of germination.

TABLE I

Chemical Composition of Germinating Castor Beans

Stage	Length hypocotyl	Ether extract	Total reducing matter as invert sugar	Glucose	Crude fiber	N × 6.25	Ash	Total	No. of lots of beans analyzed
	mm.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
0	Ungerminated	67.85	1.18		1.87	25.72	2.61	99.01	5
1	5–10	63.74	1.62		2.62	26.32	2.63	96.93	2
2	10–20	61.55	6.02		3.23	24.26	2.57	97.64	3
3	20–35	57.40	6.88		2.37	25.86	2.48	98.62	4
4	35–45	45.30	15.68		4.02	26.51	2.77	93.33	3
5	45–60	38.03	17.26		7.01	27.48	2.43	91.69	2
6	60–80	32.58	25.84	4.47	4.08	26.84	2.86	92.19	3
7	80–100	25.28	26 63	6.32	5.01	27.26	3.04	87.21	2
8	Above 100								

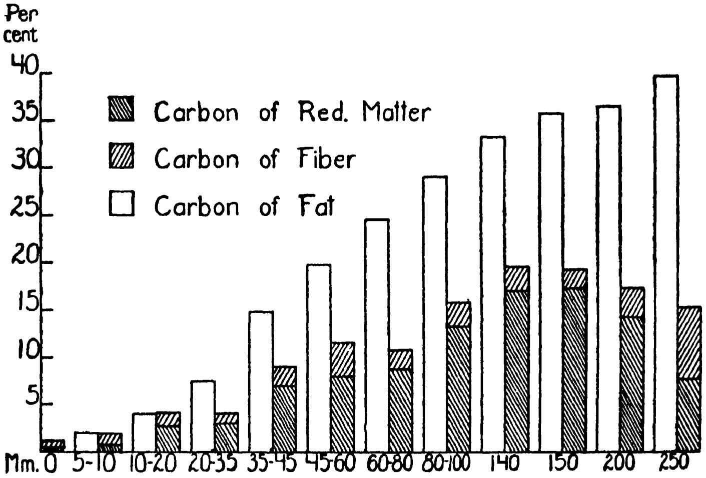

CHART 1. Carbon of fat disappearing and of carbohydrate appearing—in percentage.

In Chart I the percentage of carbon of the fat disappearing and of the reducing matter and crude fiber appearing has been calculated using the data in Table I. The fat carbon was calculated from the content in ricinolein, the carbon in the reducing matter from the content in sucrose, and in that of fiber, from the formula $C_6H_{10}O_5$. Due to the fact that we were unable to obtain 100 per cent of the constituents present in the various lots of castor beans, the fat, reducing matter, and fiber were converted to terms of 100 per cent to permit a satisfactory comparison of the results. Always the fat disappearing was determined by subtracting the percentage of fat present at each stage of germination from that present in the ungerminated seeds, corrected values, of course, being substituted for those shown in Table I. From the data in this chart it is seen that in the first two stages of germination the carbon of the fat disappearing and of the reducing matter and crude fiber appearing practically balance each other. Growth at this stage is slow, so that respiration is not as great here (see Paper I) as at some of the later periods in the development of the seedling. After these stages just mentioned, the carbon of the fat disappearing is always much greater than the carbon of the reducing substances and fiber. Much of the remaining carbon can be accounted for as that of the respiration, as will be pointed out in the discussion of the results in Tables III and IV.

TABLE II

Chemical Composition of Castor Beans in More Advanced Stages of Germination

Length hypocotyl and roots	Ether extract	Total reducing matter as invert sugar	Glucose	Crude fiber	N × 6.25	Ash	Total
mm.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
100–120	34.73	23.21	5.46	3.77	27.52	2.81	92 04
80–140	22.53	39.39	—	5.47	27.32	2.79	97 50
100–150	17.36	35.68	18 45	4.30	27.11	3.02	87.47
150–200	15.21	26 93	10 55	5.20	29.32	2.88	79.54
175–250	11.97	14.28	10.75	12 89	34.68	4.42	78.24

In Table II, the results of the analyses on seedlings, which had been permitted to germinate for a longer period than those shown in Table I, are given. These beans were kept in the dark constantly. The percentage of fat in the latest stages has now fallen to a low value. The total reducing matter of the beans with hypocotyls 80 to 140 mm.

in length calculated as invert sugar reaches a peak at approximately 40 per cent, then falls along with the fat. The reducing matter obtained prior to hydrolysis and calculated as glucose also reaches a maximum at 100 to 150 mm. length of hypocotyl and then decreases in amount. The crude fiber increases materially as would be expected.

TABLE III

Composition of Castor Beans at Beginning and End of Respiration Period

Lot No.		Ash	Ether extract	Reducing substance as invert sugar	N × 6.25	Crude fiber	Total	Undetermined
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
7	Beginning	2.31	68.48	1.21	26.75	1.08	99.83	0.17
	End	2.40	63.46	2.12	24.63	2.28	94.89	5.11
	Difference	+0.09	−5.02	+0.91	−2.12	+1.20		+4.94
8	Beginning	2.58	60.27	3.78	26.77	5.16	98.56	1.44
	End	2.61	56.00	6.38	24.68	2.97	92.65	7.35
	Difference	+0.03	−4.27	+2.60	−2.09	−2.19		+5.91
11	Beginning	2.61	67.85	1.18	25.72	1.87	99.01	0.99
	End	2.58	62.93	2.38	27.99	1.23	97.11	2.89
	Difference	−0.03	−4.92	+1.20	+2.27	−0.64		+1.90
12	Beginning	2.61	67.85	1.18	25.72	1.87	99.01	0.99
	End	2.67	63.94	2.82	29.62	1.35	100.40	
	Difference	+0.06	−3.91	+1.64	+3.90	−0.52		
13	Beginning	2.61	67.85	1.18	25.72	1.87	99.01	0.99
	End	2.38	67.72	2.60	23.63	1.24	97.17	2.83
	Difference	−0.23	−0.13	+1.42	−2.09	−0.63		+1.84
17	Beginning	2.59	57.34	10.71	24.51	1.72	96.87	3.13
	End	2.68	44.73	18.86	26.32	2.62	95.21	4.79
	Difference	+0.09	−12.61	+8.15	+1.81	+0.90		+1.66

The significance of the values given for protein is questionable as there is no certainty that the factor 6.25 is correct at all stages of the development. The ash in these beans also remains fairly constant and agrees with that of the younger stage. There is a still greater increase in the undetermined fraction.

The results as shown in Table III were obtained by analyzing beans which had been confined for various lengths of time in closed bottles as previously described. The purpose of this part of the study was to compare directly the nature of the respiratory exchange with the nature of the chemical changes as shown by analysis. It was the hope indeed to make a complete carbon and oxygen balance for the time during which the beans were confined by taking account of the composition at the beginning and end of the respiration period and the respiratory exchange itself. This hope was frustrated in part by the independent discovery of an undeterminable (by ordinary methods of proximate analysis) remainder which increased with age, in part by failure to secure 100 per cent germination or growth of the beans confined as described, and in part by the inexactness of the crude fiber method of the A. O. A. C. as applied to the material of castor beans. Nevertheless it is instructive to lay the chemical analysis side by side with the respiration figures *on the same beans.*

It is evident that the values given for the "End" in Table III cannot be put down as describing the composition of any given stage. They represent rather the mixed composition of beans which had grown unequally and some not at all. They are none the less correct analyses within the limits of the methods used.

By comparing the actual composition at the end with the average analyses of corresponding stages when the beans were confined to the respiration bottles, a difference in content is obtained which, combined with a certain amount of combustion, should account for the respiratory exchange. This obviously cannot be expected to give a perfect accounting until the nature of the undetermined material is found.

Table IV gives the respiratory exchange as found in the same lots of beans as shown in Table III. The bottles in all cases were filled with outdoor air by suction and filtration through a cotton plug, placed in the inlet tube, immediately after the beans were introduced. Hence the composition of the air at the end compared with the composition of outdoor air gave the respiratory exchange of the beans.

The respiratory quotients in all cases were between 0.48 and 0.58 and were therefore in good agreement with those shown in Table VII of Paper I, and obtained by the same method.

The changes in composition (Table III) which are characteristic in all of these cases are similar in kind, though not in amount, to those shown in Tables I and II; namely, a decrease in the percentage of ether extract, an increase in the percentage of reducing substance, and an increase of the undetermined residue. The changes in ash are negligible; those in the protein (N × 6.25) are variable, some showing an increase, others a decrease. The changes in crude fiber, which were variable in Tables I and II, though usually increasing with age, are in these special lots exceedingly variable. In four of the six lots it decreased, which we can only attribute to the fact that some of the beans, as previously mentioned, failed to germinate or to grow normally.[1]

TABLE IV

Representative Respiratory Metabolism of Several Lots of Castor Beans Whose Final Composition is Given in Table III

Experiment No.	No. of days in bottle	No. of beans	Stage in mm. length of hypocotyl		CO_2 produced	O_2 absorbed	R.Q.
			At beginning	At end			
					cc.	*cc.*	
7	3	24	1-5	Average 13.1	208	386	0.53
8	3	24	Average 15.6	" 29	326	628	0.52
11	5	21	Ungerminated	Ungerminated to 30	322	552	0.58
12	7	15	"	" " 40	302	524	0.58
13	5	20	"	" " 45	270	462	0.58
17	3	30	20-35	45-90	676	1419	0.476

The O_2 content of the bottles at the end of the respiration period in all the lots showing a decrease of crude fiber was quite low (from 0.12 per cent in No. 11 to 3.8 per cent in No. 13) but there is no parallelism between this final percentage and the decrease in crude fiber. In Lots 7 and 17, where the crude fiber increased, the percentage of O_2 at the end was relatively high (8.7 per cent in No. 7 and 5.4 per cent in No. 17).

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[1] In some other lots which had to be discarded beans were found which not only failed to germinate but degenerated, and became quite soft. It is possible that some of the ungerminated beans in the lots analyzed (Nos. 11, 12, and 13) also degenerated slightly, though they were not perceptibly softened. Softening might be due to a fermentation of the cellulose contained. The fiber therefore would escape the method of straining through fine linen used by the Association of Official Agricultural Chemists. This is only surmise.

Notwithstanding the very low final oxygens in the bottles where this occurred, most of the new growths were perfectly normal in appearance when the beans were removed and there was no odor of spoilage. Exceptionally the end of a rootlet or the point of junction of the rootlet with the hypocotyl would be darkened. It is not believed that these slight changes could alter the chemical composition or the respiratory activity.

Table V presents the results of an attempt to balance the carbon of the fat and protein (where this decreased) disappearing against the carbon of the determined amounts of invert sugar (all of the sugar being so expressed) and crude fiber formed and the C of CO_2 produced. To be perfectly fair to the experiment, the C of crude fiber has been deducted where the analysis shows a decrease. The attempt is not very successful and for this reason the balance is called a "trial balance." It is instructive only as showing the points at which the technique must be improved in future studies of this character. In Experiment 7 (first of the table), the carbon of fat and protein disappearing exceeds the carbon of sugar and crude fiber formed and of CO_2 produced, by a substantial amount. The undetermined substance indicated by the analysis as having been formed comes to 0.2833 gm., and, dividing the excess carbon by this weight, an indicated percentage

TABLE V

Trial C Balance

Experiment No.	Lost		Gained			Excess of C loss over gain	Undetermined substance	Per cent C in undetermined substance
	C of fat	C of protein	C of invert sugar	C of crude fiber	C of respiration			
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
7	0.2225	0.0657	0.0305	0.0219	0.1117	0.1241	0.2833	43 per cent (cellulose 44.4 per cent)
8	0.1813	0.0606	0.0600	−0.0533	0.1751	0.0601	0.3241	18.5 per cent
11	0.1778	—	0.0236	−0.0132	0.1730	−0.0056	0.0887	Too much CO_2 (from fermentation?)
12	0.0980	—	0.0223	−0.0075	0.1623	−0.0790	—	" "
13	0.0039	0.0414	0.0232	−0.0108	0.1451	−0.1132	0.0713	" "
17	0.897	—	0.3156	0.0268	0.3633	0.1813	0.1527	(No fermentation possible)

for the undetermined substance of 43 is obtained, which suggests a substance of about the composition of cellulose (44.4 per cent). In Experiment 8 it is possible also to calculate an excess by deducting the C of crude fiber, which decreased, instead of adding it to the right hand side of the balance; but the indicated percentage of carbon for the undetermined matter is only 18.5, which does not agree with the C content of any possible intermediary substance. Without deduction of the C of crude fiber the carbons of the two sides almost balance; but this leaves none for the undetermined residue. The next three experiments all show an excess on the side of C compounds formed, which we can only attribute to production of too much CO_2 by fermentation in the beans which failed to germinate, or having germinated, failed to grow. Experiment 17 gives a poor balance on this basis, but as will be seen below, it agrees with No. 7 on the basis of the ratio of C to O_2.

To account for the R.Q. found in Experiment 7 we may proceed as follows: We may imagine cane sugar to be formed from the ricinoleic acid by the reaction $2C_{18}H_{34}O_3 + 14\,O_2 = 3\,C_{12}H_{22}O_{11} + H_2O$. The amount of C found in the invert sugar was 0.0305 gm. (Table V). This would require $(448:432::x:0.0305)$ 0.0316 gm. O_2. Likewise we may assume that crude fiber (cellulose) is formed by the reaction $C_{18}H_{34}O_3 + 7\,O_2 = 3\,C_6H_{10}O_5 + 2\,H_2O$. The amount of C in the fiber formed was 0.0219 gm. This would require 0.0227 gm. O_2. The C of the respiration found was 0.1117 gm. If this all came from the fatty acid by the reaction $3\,C_{18}H_{34}O_3 + 75\,O_2 = 54\,CO_2 + 51\,H_2O$ it would require 0.414 gm. O_2 and would produce 0.4095 gm. CO_2, which itself would give the R.Q. $0.4095/0.414 \times 8/11 = 0.719$. Adding the oxygen necessary for formation of the sugar and cellulose we get a total of (0.414 + 0.0316 + 0.0227 = 0.4683 gm. and this would give an R.Q. of $0.4095/0.4683 \times 8/11 = 0.636$. Since the R.Q. found was 0.53, we may be certain either that some sugar was oxidized to give the CO_2 (C) found in the air or that oxygen was used also for production of the undetermined substance. There remains of the C of fat which disappeared $0.2225 - (0.1117 + 0.0305 + 0.0219) = 0.0584$ gm. and to give an R.Q. of 0.53 with the CO_2 found we require in addition to the O_2 already accounted for 0.0936 gm. Therefore it is not unreasonable to suppose this amount of oxygen has combined with

0.0584 gm. C to produce the undetermined substance, which is somewhat suggestive of arabonic acid, an oxidation product of arabinose. It is interesting to find that if sugar and cellulose formation and combustion of fat were to take place in the proportions of the reactions postulated above, *i.e.* 2 mols of $C_{18}H_{34}O_3$ to cane sugar, 1 mol to cellulose, and 3 mols for combustion, we should have 54 mols CO_2 produced and 96 O_2 absorbed which would give an R.Q. of 0.562.

If C from protein in slight amount were used to produce either cane sugar or cellulose as assumed in the trial carbon balance of Table V, the argument would not be altered and the R.Q. would not be greatly affected. If all the glycerol of the ricinolein were transformed to sugar instead of being oxidized the quotient would be diminished as indicated below.

If the entire fat molecule were oxidized, we should get:

$$C_3 H_5 (C_{18} H_{33} O_3)_3 + 78.5\ O_2 = 57\ CO_2 + 52\ H_2O$$

$$\frac{57\ CO_2}{78.5\ O_2} = 0.726 \text{ R.Q.}$$

But if the fat were first hydrolyzed, the fatty acid only oxidized, and the glycerol converted to glucose, we should have:

$$3\ (C_{18} H_{34} O_3) + 75\ O_2 = 54\ CO_2 + 51\ H_2O$$

$$2\ C_3H_8O_3 + O_2 = C_6H_{12}O_6 + 2\ H_2O$$

$$\frac{54\ CO_2}{75\ O_2 + 0.5\ O_2} = 0.715 \text{ R.Q.}$$

In the case of Experiment 17 we get the following carbon balance

C of fat disappearing		0.897 gm.
C " sugar formed	0.3156 gm.	
C " fiber	" 0.0268 "	
C " respiration	0.3633 "	0.7057
Excess C..		0.1913 gm.

Computing the O_2 necessary for formation of sugar by the reaction

$$\underset{\text{ricinoleic acid}}{2C_{18}H_{34}O_3} + 14\ O_2 = \underset{\text{cane sugar}}{3C_{12}H_{22}O_{11}} + H_2O$$

as before, we get 0.3273 gm. and for formation of crude fiber by the reaction

$$C_{18}\ H_{34}\ O_3 + 7O_2 = 3\ C_6\ H_{10}\ O_5 + 2\ H_2O$$

we get 0.0278 gm.

The oxygen necessary to form the CO_2 of the respiration (1.332 gm.) by regular combustion of the ricinolein would be 1.334 gm. with the regular R.Q. of 0.726. If we add the oxygen necessary to form the sugar and cellulose (crude fiber) found (see above) we get a total of 1.689 gm. O_2 which with the CO_2 found in the respiration bottle would give an R.Q. of $1.332/1.689 \times 8/11 = 0.574$. The actual R.Q., however, was 0.476 and with 1.332 gm. CO_2 this would require a total of 2.035 gm.[2] O_2 or a surplus of 0.346 gm. which presumably has combined with the excess carbon of 0.1913 gm. to form the undetermined substance. The proportions are again suggestive of an oxidation product of pentose.

The evidence adduced from these calculations and correlations is not at all conclusive for the chemical nature of the undetermined residue. It is presented merely as indicative of a direction in which to apply further chemical search.

Returning to the analytical results we call attention to the fairly close agreement with the analyses of Maquenne (5) and Deleano (6) also on the castor bean, and especially to their original discovery of the increasing percentage of undeterminable residue which we have confirmed. The significance of this residue in the problem of conversion of fat to carbohydrate they apparently did not appreciate. We have not been able to confirm the presence of lactic acid by Uffelmann's reagent or the thiophene reaction. No starch has been found, except in the later stages studied and then only in the hypocotyl and rootlets.

The chemistry of the germinating fatty seed is far from complete and until much more is known about it the details of the transformation of fat to carbohydrate cannot be filled in. Of the occurrence of this transformation in the castor bean, however, we no longer entertain any doubt.

SUMMARY AND CONCLUSIONS

1. Analyses for fat (ether extract), protein (N × 6.25), sugar including glucose, crude fiber, and ash have been made on all stages of the germinating castor bean up to 250 mm. length of hypocotyl and root system.

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[2] This is very close to the total actually found by analysis of the air.

2. There is a continual decrease in the amount of fat present in the whole germinating seedling, and a continual increase in the amount of sugar up to about 40 per cent (dry weight) at a hypocotyl length of 80 to 140 mm., after which it decreases as crude fiber (cellulose) increases. The most rapid decrease in fat content coincides roughly with the most rapid increase of sugar.

3. The carbon balance between fat loss and carbohydrate (including fiber) gain is not at all close, except at the very beginning of growth. An undetermined residue occurs, which increases steadily along with the total carbohydrate and accounts for more and more of the carbon.

4. The protein content which in the ungerminated bean is about 26 per cent, at first falls a little and then rather steadily increases to reach nearly 35 per cent (dry) at the last stages studied. The most plausible explanation of this is the occurrence of more and more volatile substance which is lost in drying.

5. The ash increases irregularly but in the end shows about the same ratio of increase as the protein.

6. Respiration studies on several lots of these beans at different stages of germination exhibited the same low respiratory quotients as reported in Paper I. Comparing their composition at the end of the respiration period with that of corresponding stages when the period began, the chemical change can be compared with the respiratory exchange.

7. A trial balance of all the carbon changes including the respiratory carbon and protein carbon is not very satisfactory, because of our ignorance of the undetermined residue.

8. The respiratory quotient found can be accounted for quite satisfactorily on the assumption that two out of six molecules of ricinoleic acid are converted to cane sugar, one to cellulose, and three are oxidized.

9. The oxygen needed to produce the quantities of known carbohydrate found, added to that used for combustion, and the total subtracted from the observed loss from the respired air yields in two experiments a quantity which, combined with the excess carbon, suggests that the undetermined substance may be an oxidation product of pentose.

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> of 0.9 per cent as given by Newton and Gortner. The above two varieties of wheat were non-hardy, but in the case of *Triticum vulgare* var. *Minhardi,* a hardy species, recalculation also shows a 'negative' amount of 'bound' water to be present instead of 2.2 per cent as claimed.
>
> "Obviously, then, the method employed by Newton and Gortner is unsuited for the determination of the amount of bound water in solutions and leads, in many cases, to the impossible conclusion that a *negative* amount of water is actually bound."

It must be admitted that Grollman is correct in his criticism of Formula (1) *in so far as it applies to plant saps and to solutions containing true solutes.* He was, however, unfortunate in concluding that winter-hardy wheats do not show the phenomenon of water binding, for the data which he selected for recalculation was taken from a table of data on greenhouse-grown plants and in all published work emanating from this laboratory or from the laboratories of Dr. Newton it has been expressly stated that there is no differentiation between winter-hardy and winter-tender plants grown in the greenhouse until after they have been subjected to a "hardening off" process by subjection to low temperatures for several days. Grollman's calculations were made on data taken from Table 31 (8) of tender plants. If he had recalculated the data in Table 32 (8) (*cf.* Newton (9), p. 31) his conclusions would not have been sustained. These data, together with recalculations according to Grollman's Formula (2) are shown in Table I.

In the collections of February 3 to 18 from the field we find that Super and Fulcaster wheats show no bound water. These wheats in Minnesota or Alberta are non-winter-hardy. Kanred showing 3.65 per cent of bound water is moderately winter-hardy, Minhardi with 7.49 per cent of bound water is very winter-hardy. *The order in the table from Minhardi to Fulcaster is exactly the order of winter hardiness shown by field tests,* so that the corrected Formula (2) of Grollman does not change the essential conclusion that winter hardiness in wheat is accompanied by something that is measured by this cryoscopic method and which has been designated as bound water. The greenhouse-grown wheats were demonstrated to be in a winter-tender condition and again the figures confirm this fact.

Grollman does not discuss the data for gum acacia, merely noting in the paragraph quoted "with the exception of gum acacia." It appears to us that this is a crucial exception. Here the corrected formula does not apply and the original Formula (1) of Newton and Gortner is essentially correct. In this colloidal sol appreciable amounts of bound water are shown to be present regardless of how the data are calculated.

TABLE I

The Determination of Bound Water in Certain Plant Saps and Lyophilic Sols by the Cryoscopic Method

	Material used Leaves of	Calculations by Formula (1) of Newton and Gortner					Calculated by Formula (2) of Grollman
		Δ	Δ_a	$\Delta_a - \Delta$	$\Delta_a - (\Delta + K_m)$	Bound water	Bound water
		degrees	*degrees*	*degrees*	*degrees*	*per cent*	*per cent*
Feb. 3–18, 1922. Collected from the open	Minhardi	1.741	4.226	2.485	0.400	14.4	7.49
	Buffum	1.719	4.158	2.439	0.354	13.00	5.49
	Turkey	1.273	3.612	2.339	0.254	9.7	4.10
	Kanred	1.461	3.753	2.292	0.207	8.1	3.65
	Super	1.085	3.279	2.194	0.109	4.4	−0.89
	Fulcaster	1.202	3.394	2.192	0.107	4.3	−1.60
From greenhouse. Feb. 10–16, 1922	Minhardi	1.147	3.284	2.137	0.052	2.2	−3.83
	Super	1.000	3.106	2.106	0.021	0.9	−4.37
	Cactus (stems)	0.505	2.803	2.298	0.213	8.3	5.80
	Gum acacia sols						
	1 per cent	0.005	2.147	2.142	0.057	2.37	2.32
	3 per cent	0.013	2.186	2.173	0.088	3.61	3.48
	5 per cent	0.025	2.221	2.196	0.111	4.50	4.37
	7 per cent	0.034	2.254	2.220	0.135	5.42	5.26
	10 per cent	0.048	2.294	2.246	0.161	6.39	6.15

Grollman adds that a "negative" bound water is an impossibility. This conclusion in itself is not correct for, as Bull (10) has recently pointed out, a negative bound water value is a necessary corollary when adsorption of solutes is greater than is adsorption of the solvent. If both solutes and water are adsorbed to the same extent then no bound water will be indicated.

Grollman suggests that sucrose, regarding the solutions of which we know relatively little, should be replaced by some electrolyte such as KCl, the solute behavior of which is relatively well known, and he finds in his studies of such systems little or no bound water. This finding may simply mean that the more mobile K^+ and Cl^- ions are either preferentially adsorbed or equally adsorbed along with the water, whereas the non-ionic and highly polar sucrose molecule is negatively adsorbed.

It should be added that it appears to the authors that the available data (*cf.* especially Newton and Martin (5)), showing as they do an almost exact correlation between bound water values as determined by the cryoscopic method and the biological response of plants to their environment as demonstrated by field tests, is strongly suggestive of a parallel, if not a causative, phenomenon, and this biological correlation must be accounted for before the bound water theory is thrown into the discard on what appears to be more or less theoretical assumptions.

EXPERIMENTAL

While considering certain of the problems which have been noted, an alternative method of calculating cryoscopic data occurred to us. Grollman rightly suggests that the freezing point method may be subject to appreciable errors. There are the random errors of individual determinations and there are the errors of correcting for the ice which separates due to cooling below the true freezing point. The correction for undercooling

$$\Delta = \Delta' - 0125u\,\Delta' \qquad (3)$$

where Δ = corrected freezing point
Δ' = observed freezing point
u = degrees undercooling

was used in all earlier data published from this laboratory.

Formula (3) assumes a specific heat for the system which is identical with the specific heat of water, and assumes that all of the heat of crystallization is utilized to raise the temperature of the solution. It also assumes that no heat exchange occurs between the system and its environment. Obviously these are ideal conditions which may not be realized experimentally.

It appeared, therefore, preferable to discard these assumptions and to assume only that there is a linear relationship between undercooling and the observed freezing point, which assumption appears to be well founded.

If then a series of data is secured in which are recorded the observed freezing point (Δ') and the degrees undercooling (u) such data can be treated by the method of least squares to find the constants (a) and (b) for the straight line which mathematically fits the data.

$$a = \frac{\Sigma(x) \cdot \Sigma(xy) - \Sigma(x^2) \cdot \Sigma(y)}{[\Sigma(x)]^2 - n\Sigma(x^2)} \tag{4}$$

$$b = \frac{\Sigma(x) \cdot \Sigma(y) - n\Sigma(xy)}{[\Sigma(x)]^2 - n\Sigma(x^2)} \tag{5}$$

where $x = u$

$y = \Delta'$

a = the true freezing point (*i.e.* Δ' where $u = 0$)

b = tangent of the angle which the line makes with the y axis

Such a study has been carried out on aqueous solutions of molar sucrose and M/2 KCl, KBr, and KI and on these solutions containing 3 or 5 per cent gum acacia. All solutions were *weight* molar. The gum acacia was selected from clear pieces and showed an almost negligible depression of the freezing point (0.024°) in a 5 per cent sol but the accepted true freezing points in Table II have been corrected for this slight depression. The data are shown in Table II and Figs. 1 to 4. The lines in the graphs are those calculated by the method of least squares.

DISCUSSION

Several things are evident from an inspection of the figures and Table II.

In the first place there is a relatively large probable error for an individual freezing point determination. This error is probably larger in this series of determinations than would be the case if the series were repeated. After all determinations had been completed it was observed that there was a distinct hysteresis effect observable in the acacia data. Those solutions which had stood for a time showed a slightly different freezing point scatter diagram than that shown a day or two previously by the same solution. Three series of runs (on different days) on the same solutions of sucrose + 3 per cent acacia and two series on sucrose + 5 per cent acacia are shown in Fig. 1, although all series are combined in the calculation of the constants for the line.

TABLE II

The True Freezing Point Depressions of Certain Solutions—with and without the Presence of Added Gum Acacia

System	No. of freezing points to determine line	Calculated true freezing point at $u = 0$	Angle of regression line	Change in Δ due to colloid	Average freezing point using conventional equation (3) for correcting for undercooling	Change in Δ due to colloid
		degrees	*tangent*	*degrees*	*degrees*	*degrees*
M/1 sucrose	10	2.058	0.0650		2.104	
M/1 sucrose + 3 per cent acacia	19	2.106	0.0511	+0.048	2.132	+0.028
M/1 sucrose + 5 per cent acacia	21	2.133	0.0452	+0.075	2.152	+0.048
M/2 KCl	14	1.664	0.0318		1.679	
M/2 KCl + 3 per cent acacia	16	1.640	0.0409	−0.024	1.663	−0.016
M/2 KCl + 5 per cent acacia	24	1.658	0.0343	−0.006	1.674	−0.005
M/2 KBr	10	1.699	0.0361		1.715	
M/2 KBr + 5 per cent acacia	11	1.650	0.0503	−0.049	1.679	−0.036
M/2 KI	27	1.675	0.0399		1.730	
M/2 KI + 3 per cent acacia	10	1.743	0.0213	+0.068	1.741	+0.011

That this is a hysteresis (colloid aging) effect is made certain by unpublished data on gelatin-sucrose systems secured simultaneously with those presented in this paper. Here the trend was invariably from *positive* amounts of bound water to *negative* amounts with time. This would indicate a progressive removal of sucrose from the solution by the gelatin. Unfortunately we failed to keep exact enough

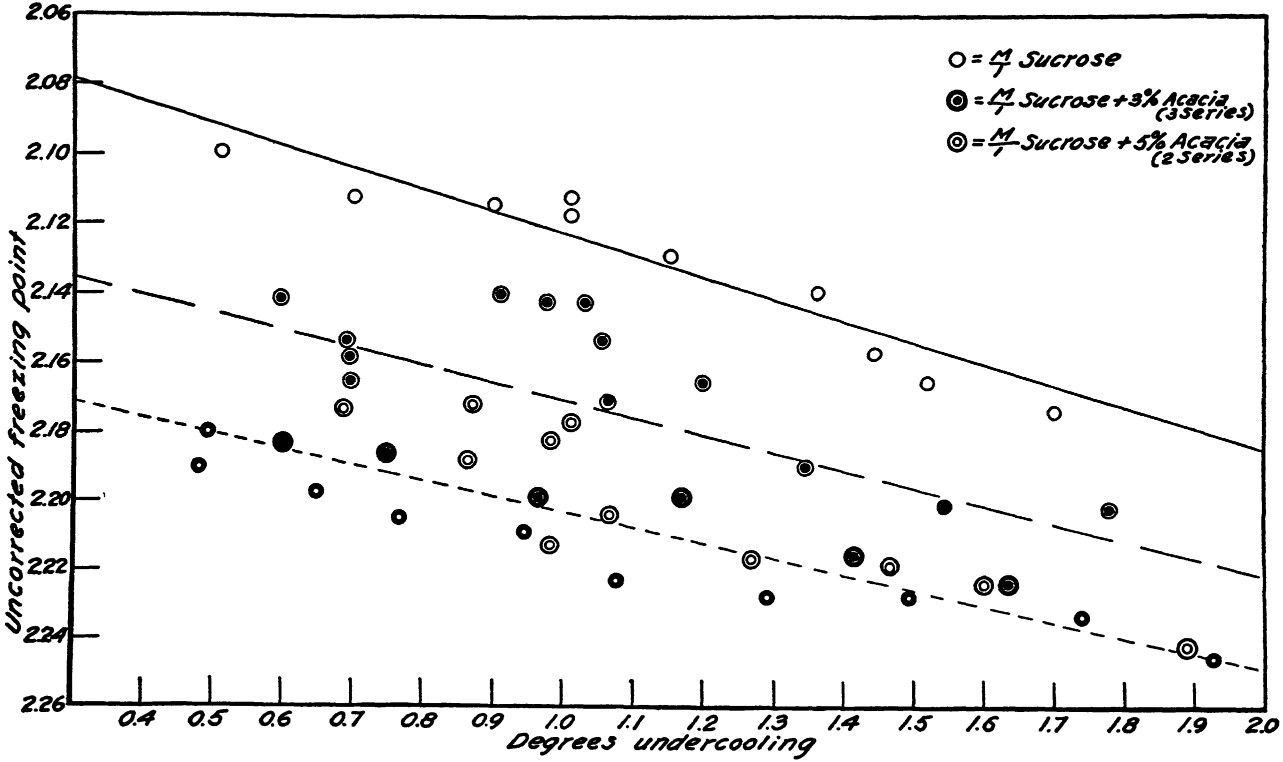

FIG. 1. Freezing point and undercooling data for M/1 sucrose solutions with and without the addition of gum acacia.

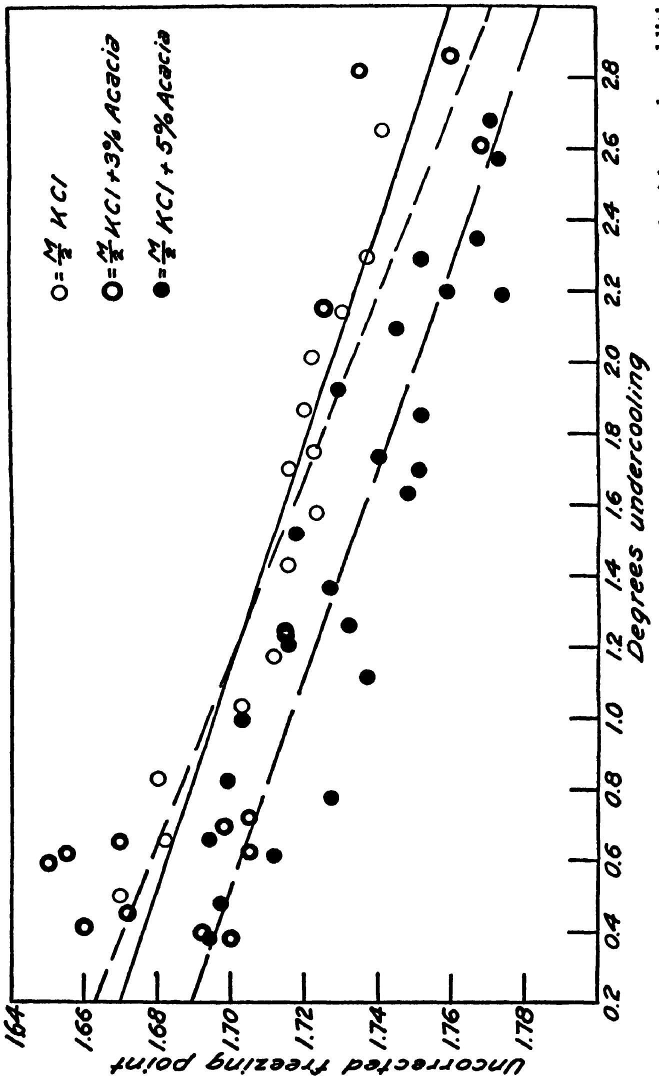

FIG. 2. Freezing point and undercooling data for M/2 KCl solutions with and without the addition of gum acacia.

records of the environmental conditions of storage of these solutions to justify publishing the data. We hope to repeat and extend the gelatin-sugar series in the near future.

In the second place it is evident that the conventional undercooling correction Equation (3) does not yield entirely correct values. This formula gives a slightly curvilinear line with tangents 0.0246 at $u = 1$ and 0.0256 at $u = 4$. The tangents found in Table II are, with only one exception, greater than these, yielding a somewhat smaller true freezing point depression (Δ) than the theoretical correction for undercooling would indicate. However, when the *difference* in freezing point depressions due to the presence of colloid are considered (Columns 5 and 7, Table II) the values derived from the two methods of calculation are remarkably consistent. We believe, however, that the least squares method, involving as it does only experimental data, is the preferable one to use on reasonably extensive series of data.

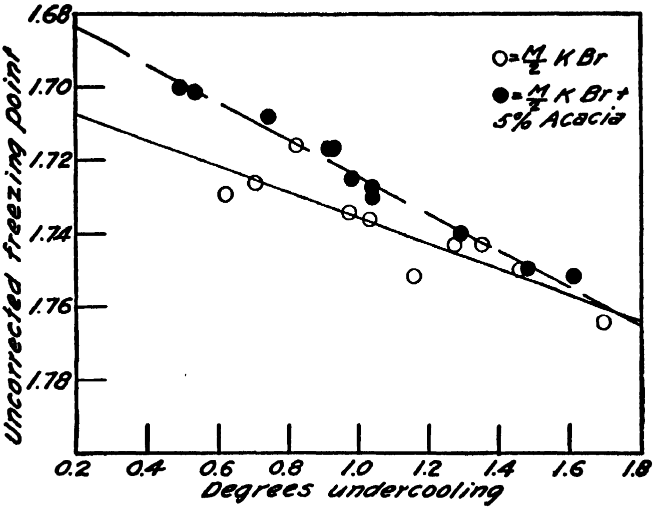

FIG. 3. Freezing point and undercooling data for M/2 KBr solutions with and without the addition of gum acacia.

In the third place the data indicate a *positive* amount of bound water in sucrose-gum acacia systems and a slightly *negative* amount in acacia-KCl and acacia-KBr systems. The acacia-KI system shows a positive value. While the values are not great, we believe that they

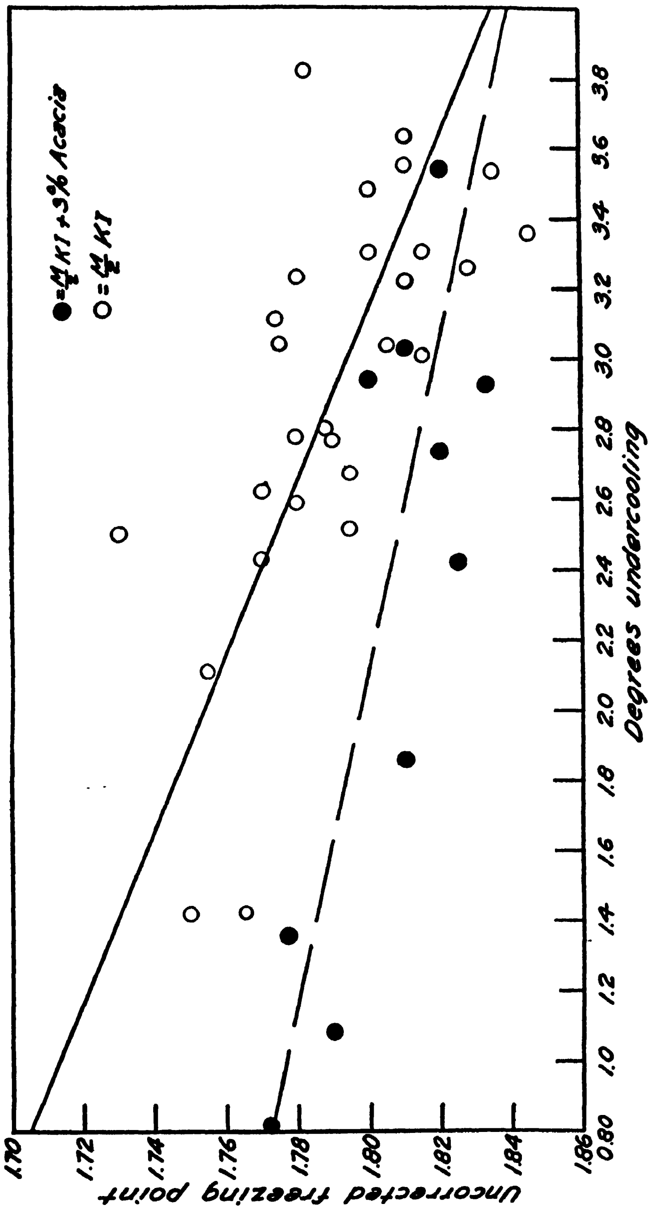

FIG. 4. Freezing point and undercooling data for M/2 KI solutions with and without the addition of gum acacia.

are significant and actually represent positive and negative adsorption of the solvent by the colloid with concomitant negative and positive adsorption of the solute; *i.e.*, the components of the solution are taken up differentially in hydrating the gum. A depression of the freezing point of 2.058° indicates a 1.106 molar solution. Therefore the gram mole of sucrose is actually dissolved in 904.2 gm. of water. This corresponds almost exactly to a pentahydrate instead of the hexahydrate of Satchard (2). The freezing point of the 3 per cent acacia-sucrose solution corresponds to a 1.132 molar solution or 1 gm. mole of sucrose dissolved in 883.3 gm. of water. The 30 gm. of gum acacia have accordingly bound 20.9 gm. of water. In a like manner the 5 per cent acacia-sucrose system corresponds to a 1.147 molar solution in which 1 gm. molecule of sugar would be dissolved in 871.8 cc. of water, with the 50 gm. of acacia binding 32.4 gm. These values are small but consistent and indicate that each gram of the colloid has bound 0.6 to 0.7 gm. of water, assuming that no sugar molecules are adsorbed. If sugar molecules are simultaneously adsorbed then the binding of water is greater. In any event the 0.6 to 0.7 gm. water per gm. of this sample of gum acacia is a *minimal* value. Newton and Gortner (1) report 3.61 per cent of bound water in a 3 per cent acacia sol and 4.50 per cent in a 5 per cent sol. These figures, applying Grollman's correction (7) are 3.48 per cent and 4.37 per cent respectively. Our present series gives 2.09 per cent bound water for a 3 per cent acacia sol and 3.24 per cent for a 5 per cent sol. Considering that we are dealing with different lots of commercial gum acacia the disagreement is not surprising. Both sets of data indicate that the colloid gum acacia takes up water preferentially from a sucrose solution, resulting in a concentration of the sucrose in the body of the liquid. The water taken up is what Newton and Gortner designated bound water.

Theoretically, if the molecular orientation hypotheses of Hardy, Harkins, Langmuir, Adam, etc., have a basis of fact, and water molecules are oriented at solid-liquid interfaces, such water molecules must be more or less immobilized and have a reduced "activity." Such immobilized molecules constitute the bound water of Newton and Gortner (1).

SUMMARY

1. The criticisms by Grollman (7) of the cryoscopic method for the determination of bound water as proposed by Newton and Gortner (1) have been considered, and it is pointed out that even admitting the correctness of his contentions does not negative the conclusion that bound water values as determined by the cryoscopic method parallel in a remarkable manner the physiological responses of plants to environmental conditions.

2. A new method of calculating the true freezing point of a solution is proposed.

3. Gum acacia in aqueous sucrose solutions shows positive amounts of bound water to the extent of 0.6 to 0.7 gm. of bound water per gram of gum.

4. Gum acacia in aqueous solutions of KCl and KBr shows slightly *negative* amounts of bound water, indicating a preferential adsorption of the solute rather than the solvent.

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THE INFLUENCE OF THE MOLECULAR WEIGHT ANTIGEN ON THE PROPORTION OF ANTIBODY TO ANTIGEN IN PRECIPITATES

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Since the study by Wu, Cheng, and Li (15) on the composition of the specific precipitate in a hemoglobin-antihemoglobin system there have appeared several papers dealing with this important problem. The antigens have included iodoalbumin (16), the pneumococcus S III hapten (5), R-salt-azo-benzidin-azo-egg albumin (6), hemoglobin (1), horse serum pseudoglobulin (10), casein-diazoarsanilic acid (8), crystalline ovalbumin (2), horse serum-diazoarsanilic acid, and others (11, 12). These studies have revealed that the composition of the precipitate is variable, depending upon the relative amounts of the two components in a mixture; also, that, in some of the systems, the ratio of antibody to antigen at the "optimal," "neutral," or "equivalence" point is fairly constant in a given system, even with sera of widely differing potencies. The reported ratios in different systems vary considerably, from 4 to 60, and in our current study of antihemocyanin (*Limulus*) we have observed a ratio as low as 1.6.

The present note is an attempt to account for the observed ratios on a simple assumption, *that at the equivalence point the antigen molecule is just completely covered by molecules of antibody.* In the cases to be discussed we suppose that the molecules of a given antigen are of the same size and are molecularly dispersed. Artificial compound antigens are omitted from consideration at present because of the, in general, inadequate experimental data, and because of our very limited comprehension of the influence of persisting unaltered or impaired native protein specificity, the effect of the number of artificial haptens per molecule and their distribution, whether on the surface or inside the molecule; information as to the specific volumes of artificial compound antigens, their state of dispersion, etc., is also lacking.

Eagle (4) has collected and contributed data which make it seem likely that antibody is closely similar to serum globulin. Svedberg's (13) studies of ultracentrifugal sedimentation indicate that the molecular weight of horse serum globulin is about three times that of egg albumin, *i.e.* 3 × 34,500; he has contributed much evidence that proteins are of two sorts, one including those built up of 1, 2, 3, or 6 units of molecular weight 34,500, and another composed of proteins of high molecular weight not obeying this rule (14). Many of the protein molecules studied by him behaved as spheres, and among the others deviations from sphericity were usually not great. Serum globulin molecules were found to be homomolecular (isometric) but non-spherical; we shall assume here that they are composed of three spheres of molecular weight 34,500, linked together in a flexible manner, so that when they are affixed to the surface of a molecule of antigen, each of the three component units is in contact with the antigenic surface.

The problem then is to ascertain how many spheres, of molecular weight 34,500, can group themselves around a given antigenic or haptenic molecule.

Since Svedberg's observations show that most proteins have practically the same partial specific volumes in solution, their volumes may be considered proportional to their molecular weights

The geometrical problem concerning the maximal number of spheres, all of the same radius, that can be brought into contact with a central sphere, which may or may not have the same radius, has apparently escaped the attention of mathematicians. The following formula is believed to be a very close approximation.

$$N = 2 + \frac{90^\circ}{\tan^{-1} \sqrt{\tan\left(\frac{3\theta}{2}\right) \tan^3\left(\frac{\theta}{2}\right)}}$$

where N = the number of outer spheres, ρ = the ratio of the radius of the outer spheres to that of the inner sphere, and $\sin\theta = \frac{\rho}{1 + \rho}$.

Then, N times 34,500, divided by the molecular weight of the a gives the predicted ratio by weight. If the "antigen" is not a protein, suitable correction—which will be small—for the difference in specific volume should be considered.[1]

[1] The derivation of this formula is as follows: consider three outer spheres of radius r', in mutual contact, on the surface of the inner sphere with radius r and center at O. Fig. 1 represents the section by a plane through the centers of the inner and two of the outer spheres. The points of contact A, B, and C of the outer spheres with the inner sphere determine the vertices of an equilateral spherical triangle ABC on the surface of the inner sphere. It is, of course, impossible to show point C in the two-dimensional Fig. 1. The area of this spherical triangle is not completely covered by the three outer spheres, but since this is the closest packing possible, we may say that the area of this triangle, divided by the sum of the portions cut out of the three outer spheres by planes ABO, ACO, BCO (represented in the schematic Fig. 2 by shaded areas), gives the area which *one* outer sphere can *effectively* cover.

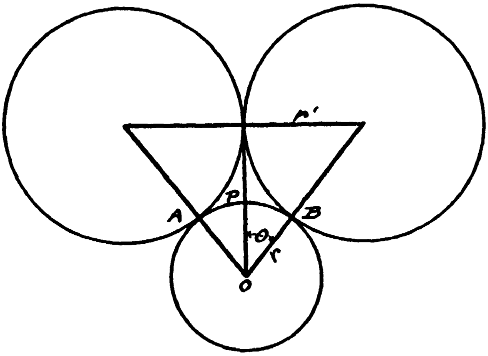

FIG. 1

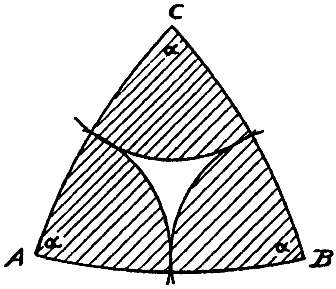

FIG. 2

This included portion will be 3 × $\alpha/360°$, where α = the angle of the triangle, which can be computed by l'Huilier's formula giving the relation between the spherical excess and the sides

$$\tan E/4 = \sqrt{\tan\left(\frac{3\theta}{2}\right)\tan^3\left(\frac{\theta}{2}\right)}$$

where E = the spherical excess of the triangle (sum of angles − 180°) and θ = the angle POB, $\sin\theta = r'/(r + r') = \rho/(1 + \rho)$, where $\rho = r'/r$; so that

$$\alpha = 60° + 4/3 \tan^{-1}\sqrt{\tan\left(\frac{3\theta}{2}\right)\tan^3\left(\frac{\theta}{2}\right)}$$

(*Footnote continued on following page*)

When one deals with single antigens, the Dean and Webb (3) optimum, at least in some systems, indicates essential neutrality (8, 12); at this point the supernatant fluid is either devoid of demonstrable antigen and antibody, or *both* are present in small amounts, which vary presumably with the dissociation constants of the compounds. At this point, the precipitate in a crystalline ovalbumin system was found to be maximal, or nearly so (2). Precipitates formed in the region of antigen excess would not be expected to obey our rule.

The area *effectively* covered by one outer sphere ($A_{\mathrm{eff.}}$), will be $\frac{A}{3\alpha/360^\circ}$ where A = the area of the spherical triangle = $\pi r^2E/180^\circ$. Substituting, this becomes

$$A_{\mathrm{eff.}} = \frac{\pi\, 8r^2 \tan^{-1} \sqrt{\tan\left(\frac{3\theta}{2}\right) \tan^3\left(\frac{\theta}{2}\right)}}{3\left[60^\circ + 4/3 \tan^{-1} \sqrt{\tan\left(\frac{3\theta}{2}\right) \tan^3\left(\frac{\theta}{2}\right)}\right]}$$

The number of outer spheres, N, will be given by $4\,\pi\, r^2/A_{\mathrm{eff.}}$, where $4\,\pi\, r^2$ = the area of the inner sphere. Substituting for $A_{\mathrm{eff.}}$,

$$N = 2 + \frac{90^\circ}{\tan^{-1} \sqrt{\tan\left(\frac{3\theta}{2}\right) \tan^3\left(\frac{\theta}{2}\right)}}$$

In applying this formula, we assume that the volumes of different protein molecules are proportional to their molecular weights, so that $\rho = \sqrt[3]{M'/M}$. When the antigen is not a protein the expression M'/M is multiplied by the ratio of the specific volumes. Taking the carbohydrate S III as an illustration, we have, assuming its specific volume to be the same as that of sucrose,

$$\rho = \sqrt[3]{\frac{34{,}500 \times 0.75}{4{,}000 \times 0.64}} = 2.15.$$

Therefore $\sin\theta = 2.15/3.15$, and $\theta = 43^\circ\ 2.57'$. Then

$$N = 2 + \frac{90^\circ}{\tan^{-1} \sqrt{\tan 63^\circ\ 49' \tan^3 21^\circ\ 16.29'}} = 2 + 4.72 = 6.72$$

and the ratio by weight of antibody to antigen would be 6.72 × 34,500/4,000 = 58.

The L-hemocyanin we have used was kindly furnished by Professor Redfield and is considered to be of high purity; it was repeatedly precipitated at its isoelectric point; different samples thus prepared have given nearly identical analytical figures. The equivalence point was determined by that optimal particulation method in which antigen is the variable, and we measured the nitrogen present in the precipitate obtained in the zone of slight antibody excess.

TABLE I

Ratio by Weight of Antibody to Antigen

Antigen	Molecular weight	Calculated ratio	Observed ratio	Source of data
Pneumococcus S III	4,000	58	60	(5)
Egg albumin	34,500	13.4	13	(2)
Hemoglobin	68,000	9.7	10	(15)
Pseudoglobulin	103,800	7.3	4	(10)
L-hemocyanin	2,000,000	1.47	1.57	(9)

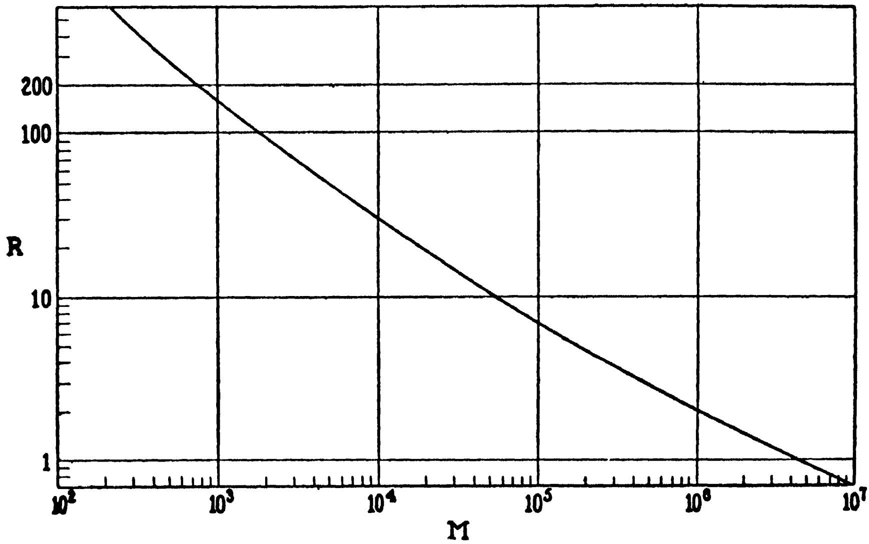

FIG. 3. Calculated relation between ratio by weight (R) and molecular weight of antigen (M).

The table gives the comparison between calculated and observed ratios. The number of systems concerning which data are available is small, but practically the extremes of molecular weights of antigens are represented, together with some of the important intermediates.

The graph provides a means of predicting the ratio to be expected when one is using an antigen of known molecular weight, and, if the assumption is correct, one might approximately estimate the molecular weight of any pure antigen or precipitable hapten.

DISCUSSION

Since the present accuracy obtained in determinations of antibody-antigen ratios did not seem to demand it, we have here taken no account of the fact that, in nature, N of course should always be an integer—and presumably also a multiple of 3, since globulin $= 3 \times 34{,}500$; instead we have used without modification the results of the formula in which N is a continuous function of ρ. If globulin is assumed to be a single sphere there are large discrepancies between observed and calculated ratios. The latter are too high. Possibly there is some flattening of the adsorbed antibody molecules which would account for this, but we are too ignorant of the subject of molecular distortion to deal with it in this connection.

The molecular weight of 4,000 for S III is chosen as a fair mean of the values obtained by Heidelberger and Kendall by various methods (7).

The figures for hemocyanin, in spite of the close check, should not be given too great weight, as our work on that system is incomplete. Due apparently to the fact that the precipitate is more dissociable than those from other systems, comparatively large corrections had to be applied to the analytical figures. Analysis for copper in the precipitate indicated that only about 90 per cent of the hemocyanin was thrown down in this zone of slight antibody excess. The fact that L-hemocyanin was not found to be exactly spherical is probably of less importance.

The hemoglobin ratio, derived from the observations of Wu, Cheng, and Li, is assigned only tentatively. They did not exactly determine the point of equivalence but it would appear that their measurements were made in a zone closely neighboring neutrality. Some of Breinl and Haurowitz' (1) data are similarly concordant.

The greatest discrepancy occurs in the case of pseudoglobulin.

It seems possible to us that the low observed ratio could be due to failure of a part of the added protein antigen to precipitate; we had this informative experience in connection with as yet unfinished work on crystalline lactalbumin. It should be emphasized that the assumption that all of a portion of protein antigen added to an excess of antiserum enters into the precipitate is, in the absence of a chemical "marker" on the added protein, extremely perilous. One has no assurance that all of the antigenic "nitrogen" is immunologically active. Further, in view of the difficulty of separating the serum globulins and of preparing specific "univalent" sera for them, it is quite possible that the globulin preparation, although entirely active, may be but partially homologous to the antiserum employed. Indeed, in globulin systems, we have repeatedly observed two optima between which antigen was present in large excess in the supernatant fluids. We were therefore unable to establish any reliable antibody-antigen ratio.

In addition, our own calculations regarding pseudoglobulin, as antigen, are none too consistent. The ratio 7.3 is arrived at by considering the molecule to be spherical. When computations are based on the assumption that it is an aggregate of three spheres (3 × 34,500) the ratio is 10 or 11 depending upon how the antibody spheres are supposed to orient themselves around the antigen. The situation is decidedly unsatisfactory, but demands discussion.

A further substantiatory indication of the influence of antigenic molecular weight upon the antibody-antigen ratio is afforded by the relative magnitudes of limiting titres determined by the antigen-dilution method. The notoriously active pneumococcus polysaccharides still react visibly when diluted several million fold; hemocyanin, according to our observations, had a limiting titre of about 300,000 in tests with sera of an antibody concentration (2 to 3 mg./ml.) similar to that possessed by many antipneumococcus sera. It is obvious that progressive dilution would reduce the number of molecules below the effective precipitating concentration (assuming equal dissociation constants) much sooner in the case of hemocyanin than with S III, an equivalent weight of which would contain some 500 times as many molecules.

SUMMARY

The assumption that, at the equivalence point in specific precipitin reactions, the antigen molecule is completely covered with a single layer of antibody-globulin molecules has been shown to account fairly well for the antibody-antigen ratios of some representative native single proteins, and the pneumococcus S III hapten.

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BACTERIAL CELL METABOLISM UNDER ANAEROBIC CONDITIONS*

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Objectives

Since the days of Pasteur it has been known that under anaerobic conditions microorganisms display special powers of attacking carbohydrate molecules. As a rule, however, such observations have been made on mass cultures with no knowledge of the actual number of cells concerned. In the ordinary routine bacteriological tests for fermentation, even the amount and type of gas produced is only conjectural since the changing reaction of the medium influences to an unknown degree its power to absorb gaseous substances and to diffuse them from its free surface into the atmosphere. Where this error has been avoided experiments have usually been conducted in closed vessels in the presence of accumulating waste products of metabolism which produce highly abnormal conditions.

In other contributions from this laboratory (Walker and Winslow, 1932; Walker, Winslow, Huntington, and Mooney (1934)), data have been presented with regard to the metabolic activity of *Escherichia coli* in media continuously aerated with air which had previously been freed from ammonia and carbon dioxide. Simultaneous determinations of the population present made possible computations of yield per cell per hour. Such a method of cultivation presents far more favorable conditions for the study of bacterial metabolism than the ordinary procedure of growing them in closed flasks, since it removes at least the volatile waste products of their life processes. It was found, in the first study cited, that the addition of lactose to a peptone water medium caused a marked decrease in the yield of ammonia (per cell per hour) but that the yield of carbon dioxide remained substantially unaffected.

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In the second of the two papers cited we showed that the effect of glucose was the same as that of lactose. The addition of this sugar to the peptone water medium again showed a protein-sparing action; but the yield of carbon dioxide per cell was also reduced,—although much less so than the yield of ammonia. Thus, in a medium rendered highly aerobic by bubbling air through it, metabolic activity as measured by CO_2 production remains substantially unaffected by the addition of carbohydrates.

The purpose of the present study was to obtain similar quantitative data for cultures maintained under anaerobic conditions by bubbling through the media nitrogen instead of air.

Technique of Present Study

The culture employed in all our work was the same strain of *Escherichia coli* used in previous studies. The media were peptone water (1 per cent Difco-Bacto peptone) and glucose peptone water (1 per cent Difco-Bacto peptone plus 0.5 per cent Baker's C.P. glucose). In our routine experiments, the bacteria, washed off from 12–18 hour agar slants were filtered through paper and added to the medium in Dreschel bottles to give an initial count of about 10 million organisms per cc. Duplicate inoculated bottles and a third uninoculated control were placed in a water bath at 37°C. The bottles were connected with the CO_2-absorbent portion of the aeration train of Walker as modified by Walker, Winslow, Huntington, and Mooney (1934). Instead of the air used in the earlier experiments, nitrogen gas was bubbled through the bottles for half an hour before inoculation and then for 6 hours after inoculation. The gas was delivered from a cylinder of nitrogen provided with a needle valve and on the culture inlet side of the train was placed as before a Milligan spiral gas-washing bottle filled with KOH for the absorption of CO_2, and the other purification train units,—H_2SO_4, wash water, and indicator.

Portions were withdrawn from the medium each hour for plating and for the determination of the carbon dioxide content of the medium by the use of the fine-bore blood gas apparatus of Van Slyke and Stadie (1921); while the carbon dioxide carried off in the gas train was quantitatively absorbed by a Brady-Meyer absorption tube and controlled by a Bowen potash bulb,—both containing $Ba(OH)_2$. The methods of absorption and determination are described in another paper (Walker, Winslow, Huntington, and Mooney (1934)). The technique was in all respects the same as that employed in previous studies except for the replacement of air by nitrogen in the gas train and except for the fact that the experiments were continued for 6 hours instead of 4 or 5.

RESULTS

Our new results with the use of the nitrogen train are presented in Tables I and II. The figures for bacterial numbers and for cumulative yield of CO_2 are in all cases the average of eight different determinations. The individual tests checked each other reasonably well, as indicated by the probable errors of the means in the table. For bacterial counts, the probable errors were generally about one-tenth of the mean values and for CO_2 increments generally less than one-third the mean values. These variations do not affect the conclusions to be drawn. The yields per cell per hour in the last column were

TABLE I

Growth and Carbon Dioxide Yield in Peptone Water through Which Nitrogen Was Continuously Bubbled

Age	Bacteria, *millions per cc.*		CO_2 yield		
	Actual	Log	Increment	Cumulative total	Rate per cell per hr. during previous interval
hrs.			*mg. per 100 cc.*	*mg. per 100 cc.*	*mg.* × 10^{-11}
0	11.4 ± 1.2	1.057			
1	10.4 ± 1.2	1.017	0.40 ± 0.17	0.40	42
2	10.4 ± 0.8	1.017	0.28 ± 0.07	0.68	27
3	18.8 ± 2.4	1.274	0.95 ± 0.16	1.63	68
4	28.8 ± 2.8	1.459	0.53 ± 0.22	2.16	22
5	32.3 ± 5.2	1.509	0.48 ± 0.18	2.64	15
6	38.3 ± 4.7	1.583	0.51 ± 0.08	3.15	15

TABLE II

Growth and Carbon Dioxide Yield in Glucose Peptone Water through Which Nitrogen Was Continuously Bubbled

Age	Bacteria, *millions per cc.*		CO_2 yield		
	Actual	Log	Increment	Cumulative total	Rate per cell per hr. during previous interval
hrs.			*mg. per 100 cc.*	*mg. per 100 cc.*	*mg.* × 10^{-11}
0	11.7 ± 1.0	1.068			
1	11.1 ± 1.2	1.045	0.47 ± 0.14	0.47	42
2	12.7 ± 1.4	1.104	0.44 ± 0.21	0.91	36
3	30.5 ± 6.3	1.484	2.04 ± 0.65	2.95	87
4	66.1 ± 14.6	1.820	9.52 ± 2.18	12.47	211
5	142.4 ± 28.6	2.153	14.79 ± 1.13	27.26	188
6	151.7 ± 20.6	2.181	12.00 ± 1.71	39.26	94

obtained by the formula of Buchanan (1918) as employed by Walker and Winslow (1932).

Influence of Air and of Sugar upon Growth

The pertinent data with regard to bacterial multiplication are brought together for comparison in Table III and in Fig. 1. The columns headed "Air" are from the data obtained by Walker, Winslow, Huntington, and Mooney (1934) for cultures through which air was bubbled; the columns headed "Nitrogen" are from Tables I and II of the present paper.

TABLE III

Bacterial Growth in Peptone Water and Glucose Peptone Water through Which Either Air or Nitrogen Was Bubbled Continuously

Age	Bacteria, *millions per cc.*			
	Air		Nitrogen	
	Peptone	Glucose peptone	Peptone	Glucose peptone
hrs.				
0	15.9	17.3	11.4	11.7
1	14.5	17.1	10.4	11.1
2	22.9	44.5	10.4	12.7
3	85.3	172.0	18.8	30.5
4	236.0	585.0	28.8	66.1
5	655.0	861.0	32.3	142.4
6			38.3	151.7

It will be noted that the cultures grown in a peptone water medium through which nitrogen was bubbled showed a long lag period of 2 hours and then a very slow rate of increase amounting during the period from the 2nd to the 5th hour only to a tripling of the bacterial population. Clearly, the organism studied is here markedly inhibited by anaerobic conditions. The addition of glucose to this medium (still with a continuous current of nitrogen) improved matters considerably. The lag period was again prolonged; but between the 2nd and the 5th hours the population increased more than tenfold. The presence of a fermentable carbohydrate as one would expect makes the peptone medium more satisfactory for anaerobic cultivation.

The media saturated with air instead of nitrogen are, however, far more favorable. The lag period lasts only 1 to 2 hours; and the increase from the 2nd to the 5th hour is 20- to 30-fold. The peptone glucose medium is again more favorable than that containing peptone alone; but the difference is relatively slight.

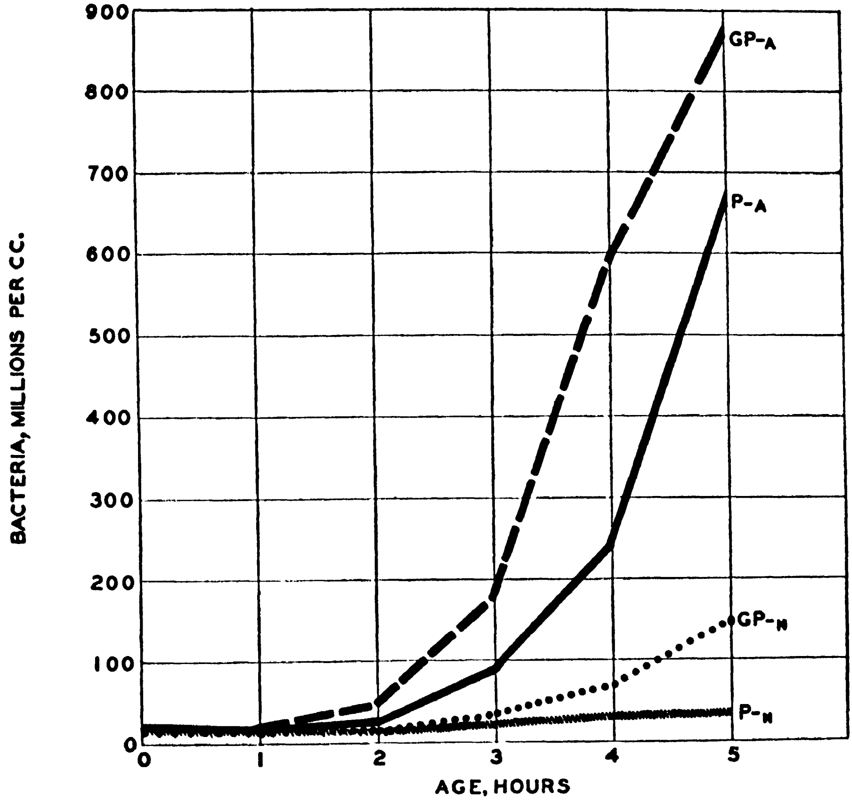

FIG. 1. Bacterial counts under various conditions. *GP-A* Glucose peptone medium through which air was bubbled. *P-A* Peptone water medium through which air was bubbled. *GP-N* Glucose peptone medium through which nitrogen was bubbled. *P-N* Peptone water medium through which nitrogen was bubbled.

Influence of Air and of Sugar upon Metabolism

Table IV and Fig. 2 present corresponding data for the yield of CO_2 computed per cell per hour.

It will be noted that the bacteria in the media saturated with air behave in essentially the same fashion whether glucose be present or not. They show a sharp rise in the rate of CO_2 yield during the 2nd hour, followed by a steady fall. This is the phenomenon recorded in all our previous work.

When the medium is saturated with nitrogen instead of air there is a very prolonged metabolic lag period, both in the presence and in the absence of sugar. The rate of CO_2 production decreases slightly, instead of increasing greatly, during the 2nd hour. During the 3rd hour, however, there is a slight increase.

In the peptone medium, under these anaerobic conditions, the yield of CO_2 only reaches 68 mg. × 10^{-11} per cell per hour during the 3rd hour (about one-half the value recorded for the maximum hour in the aerobic cultures) and then falls off.

TABLE IV

Carbon Dioxide Yield in Peptone Water and Glucose Peptone Water through Which Either Air or Nitrogen Was Bubbled Continuously

Age	Rate per cell per hr. during previous interval			
	Air		Nitrogen	
	Peptone	Glucose peptone	Peptone	Glucose peptone
hrs.	*mg.* × 10^{-11}	*mg.* × 10^{-11}	*mg.* × 10^{-11}	*mg.* × 10^{-11}
1	37	43	42	42
2	123	117	27	36
3	73	63	68	87
4	50	35	22	211
5	22	16	15	188
6			15	94

In the anaerobic medium containing glucose a fundamental change in metabolism sets in after the 3rd hour. The yield of carbon dioxide per cell per hour, instead of falling off as in the peptone medium, shoots up to the enormous value of 211 mg. × 10^{-11} per cell per hour. Clearly, at this point true anaerobic decomposition of the carbohydrate is going on, at a rate nearly double the maximum recorded in any of our aerobic experiments.

It has been pointed out in another communication (Walker, Winslow, Huntington, and Mooney (1934)) that variations in metabolic yield per cell per hour may in part be accounted for by variations in cell volume at different phases of the life cycle. This problem we are now studying. In the present instance, another possible factor suggested itself. Might the high yields of carbon dioxide per cell in the

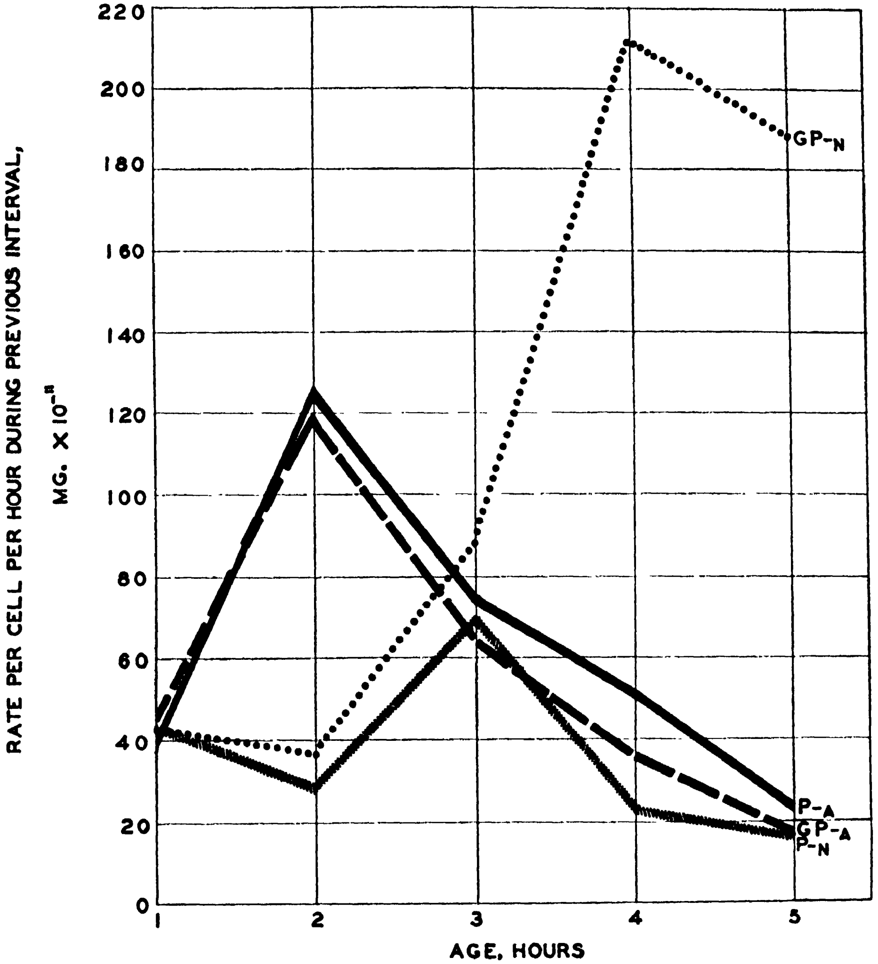

FIG. 2. Yield of CO_2 per cell per hour. *GP–A* Glucose peptone medium through which air was bubbled. *P–A* Peptone water medium through which air was bubbled. *GP–N* Glucose peptone medium through which nitrogen was bubbled. *P–N* Peptone water medium through which nitrogen was bubbled.

anaerobic peptone glucose medium be due to wholesale death of the bacteria and liberation of CO_2 from dead and dying cells which would not, of course, appear in the plate counts? This seemed inherently unlikely; but the possibility was suggested in part by an apparent increase in culture turbidity adjudged from long experience to be in excess of the population growth as shown by plating. To determine whether such was the case, check determinations were made by direct microscopic counts, using the differential stain of Proca-Kayser (Kayser, 1912) which is designed to distinguish between living and dead cells. These control determinations checked closely with the plate counts and also showed an inappreciable number of dead cells to be present.

CONCLUSIONS

Escherichia coli has been cultivated in a peptone water medium saturated continuously with nitrogen by use of a gas train so as to produce anaerobic conditions. Under these circumstances growth was greatly inhibited. Cultures which originally contained 11 million bacteria per cc. showed on the average only 32 million after 5 hours (as compared with 655 million in similar cultures saturated with air).

The metabolic activity of the cells in such a culture was greatly reduced by the anaerobic conditions. It actually fell off from 42 mg. $\times 10^{-11}$ per cell per hour during the 1st hour to 27 mg. during the 2nd hour and rose only to a maximum of 68 during the 3rd hour. Similar cultures saturated with air showed a rise from 37 mg. $\times 10^{-11}$ during the 1st hour to 123 during the 2nd hour.

The addition of glucose to the medium, under aerobic conditions, has been shown in previous studies to cause only a slight increase in bacterial numbers (861 instead of 655 million after the 5th hour). In the cultures aerated with nitrogen, the addition of glucose has no effect during the first hours. There is again a long lag period and a reduced metabolic rate. After the 2nd hour, however, a wholly different phenomenon manifests itself. The bacterial population increases more rapidly than in the anaerobic peptone medium (reaching a maximum of 142 million after 5 hours). This growth is accompanied by an enormous increase in the rate of CO_2 yield, which reaches 211 mg. $\times 10^{-11}$ per cell per hour during the 4th hour (nearly double the maximum values recorded under aerobic conditions). The same phenomenon is, of course, illustrated by the enormous yield of CO_2 produced by the action of fermenting organisms in carbohydrate

THE CRYOSCOPIC METHOD FOR THE DETERMINATION OF "BOUND WATER"*

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In 1922 Newton and Gortner (1) put forward the hypothesis that at least a portion of the water associated with the hydrophilic colloids in plant tissues was in a "bound" form, in which form it exhibited physicochemical properties which would serve to differentiate it from the "free" water of the vacuolar sap. It was suggested that bound water might be unavailable for the solution of sucrose and accordingly a method for the estimation of such bound water was proposed which consisted essentially in first determining the freezing point of the solution which contained the hydrophilic colloid and denoting this value as Δ. An amount of the sol was then taken which contained exactly 10 gm. of total water. To this portion there was added 0.01 mole of sucrose and a second depression of the freezing point, designated Δa, was determined. If all of the 10 gm. of water were free to dissolve sucrose Δa should differ from Δ by the freezing point depression of a molar solution of sucrose which according to Satchard (2) should be 2.085°, corresponding to a gram molecule of sucrose-hexahydrate dissolved in 892 gm. of water.

The formula which Newton and Gortner proposed

$$\text{percentage of bound water} = \frac{\Delta a - (\Delta + Km)}{\Delta a - \Delta} \times 89.2 \qquad (1)$$

was tested by them on aqueous solutions of gum acacia and on certain plant saps and appreciable amounts of bound water were apparently demonstrated to be present.

This method has been extensively used by numerous plant physiologists—especially Newton and his coworkers (3–6)—and has

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apparently given valuable information as to the physiological response of plants, especially in problems concerned with winter hardiness and drought resistance.

Bound water appeared to be more or less generally accepted as a physiological factor and the cryoscopic method as an approximate[1] measure of its intensity. However, Grollman (7) has recently challenged the conception of bound water and the correctness of the formula proposed by Newton and Gortner (1).

Grollman suggests that Formula (1) does not provide for the concentration of electrolytes and other true solutes which are initially present in the biological fluids and that if sucrose forms a hexahydrate these solutes must be concentrated in the remaining water, thus forming a more concentrated solution which will freeze below the Δ as originally determined on the sap. He accordingly proposes the formula

$$\text{percentage of bound water} = \frac{\Delta a - \frac{1000}{892}\Delta + Km}{\Delta a - \frac{1000}{892}\Delta} \times 89.2 \qquad (2)$$

as a measure of bound water, and adds:

> "This modification of Newton and Gortner's method of calculation will markedly affect results quoted by these authors in which Δ is appreciable compared to Δ*a* and K*m* as is the case in practically all of the substances studied by these authors with the exception of gum acacia. In the latter case, the correction is comparatively slight.
>
> "Newton and Gortner's results by the above method, as applied to the juices of the wheat plant (*Triticum vulgare*) led these authors to conclude that winter hardiness in such plants is related to the amount of bound water present in different varieties. If one corrects their results by the use of the modified formula described above, one finds very little evidence to substantiate this theory. Thus in the case of *Triticum vulgare* var. *super*, in which 4.4 per cent of the water was found to be bound, recalculation shows the results to indicate that all the water is actually free. Recalculation of the results for a second species of the same plant shows −3.6 per cent of the total water to be bound (an obviously impossible result) instead

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[1] "Approximate" only, since the method *must* yield *minimum* values as the assumptions are made (*a*) that bound water does not "dissolve" sucrose, *i.e.* water is adsorbed but no sucrose is adsorbed, and (*b*) that a molar solution of sucrose in water does not shift the bound ⇄ free water equilibrium.

media recorded by Anderson (1924) and other students of the obligate anaerobes. We have here, however, a somewhat striking illustration of the distinct type of metabolic activity manifested by a facultative organism under anaerobic conditions in the presence of sugar measured on a cell-per-hour basis. This is a quantitative illustration of the "life without air" described by Pasteur.

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CRYSTALLINE PEPSIN

VI. Inactivation by Beta and Gamma Rays from Radium and by Ultra-Violet Light

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(Accepted for publication, September 28, 1933)

Previous work (1) has shown that when solutions of crystalline pepsin are inactivated by alkali or by heat the loss in activity is exactly proportional to the loss of native protein. These experiments confirm the idea, therefore, that the native protein molecule is the active enzyme. Proteins are denatured (2) by exposure to radium or to ultra-violet light and it is also known that pepsin solutions (3) are inactivated under these conditions. Inactivation of the enzyme by radium or ultra-violet light, therefore, furnishes another method of testing the relationship between the protein and the active molecule. If the protein molecule itself is responsible for the activity then any loss in activity must be accompanied by a corresponding decrease in the protein. On the other hand, if a hypothetical, active molecule is merely associated with the native protein there is no reason to suppose that the rate of inactivation of the active molecule would be the same as the rate of denaturation of the protein. The inactivation of pepsin solutions has been studied from this point of view and it has been found that the loss in activity is just proportional to the loss in native protein when the enzyme is inactivated either by radium or by ultra-violet light. These results, therefore, furnish additional evidence in favor of the idea that the protein molecule itself is responsible for the activity.

EXPERIMENTAL RESULTS

I. Decrease in Activity of Protein Nitrogen of Pepsin Solutions Exposed to Radium Bromide at pH 5.0 and 0°C.

The results of an experiment in which pepsin solutions of various concentrations were exposed to the radiation of 100 mg. of radium bromide at 0°C. are shown in Table I. The decrease in activity, as determined by the hemoglobin method, is just proportional to the decrease in the protein nitrogen of the solution as shown by the fact that the activity per mg. protein nitrogen remains constant. No denatured protein appears in the solution although it is probable

TABLE I

Changes in Activity and Protein Nitrogen of Pepsin Solutions Exposed to Radium Bromide, pH 5.0, 0°C.

hrs.									
0	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$	0.5	0.2	0.043	0.048	0.010	0.010	0.0048	0.0048
	P N/ml., *mg*	2.6	1.0	0.24	0.22	0.050	0.050	0.023	0.023
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$	0.19	0.20	0.21	0.22	0.20	0.20	0.21	0.21
25	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$							0.0041	
	P N/ml., *mg*							0.020	
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$							0.20	
72	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$			0.037					0.0036
	P N/ml., *mg*								0.019
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$								0.19
96	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$				0.031		0.0054		
	P N/ml., *mg*				0.16		0.030		
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$				0.19		0.18		
160	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$	0.5		0.021		0.0056			
	P N/ml., *mg*	2.5		0.10		0.035			
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$	0.20		0.21		0.15			
190	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$		0.17						
	P N/ml., *mg*		0.78						
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$		0.22						
310	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$	0.43							
	P N/ml., *mg*	2.17							
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$	0.20							
770	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$		0.083						
	P N/ml., *mg*		0.48						
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$		0.17						

that the first step in the reaction is the formation of denatured protein. The rate of denaturation under these conditions, however, is extremely slow and the denatured protein, if present, would undoubtedly be digested by the remaining active native protein as rapidly as it was formed and so does not accumulate in the solution.

Effect of the Concentration of Pepsin

Below about 0.05 mg. nitrogen per ml. the per cent inactivated is nearly constant, while in more concentrated solutions the actual quantity inactivated is approximately constant. Similar results were obtained by Hussey and Thompson (3). They indicate that, under the conditions of the experiment most of the energy is absorbed by 0.05 mg. nitrogen per ml. so that increasing the concentration beyond this point does not have much effect upon the number of protein molecules inactivated.

Experimental Procedure

A solution of three times crystallized pepsin was prepared in pH 5.0 N/20 acetate buffer and diluted to the concentrations noted in the table with N/20 acetate buffer. 25 ml. of this solution was placed in a 1.5 cm. centrifuge tube and a glass tube (with 0.5 mm. walls) containing 100 mg. of radium bromide suspended in the center of the solution. The tube was kept in the ice box at 0°C. and 1 ml. samples removed and analyzed for protein nitrogen and activity as noted in the table.

Activity Determinations.—The activity determinations were made with hemoglobin by the method of Anson and Mirsky(4).

Protein Nitrogen Determination.—1 ml. of solution added to 5 ml. of 5 per cent boiling trichloracetic acid, the precipitate centrifuged and washed three times with 5 per cent trichloracetic acid, and total nitrogen in the precipitate determined.

Test for Denatured Protein.—1 ml. of solution added to 10 ml. of N/2 sodium sulfate and N/20 sulfuric acid. Any denatured protein precipitates under these conditions and some of the samples which had been almost completely inactivated gave a slight cloud. The amount of denatured protein was, however, too small to determine.

II. Inactivation by Ultra-Violet Light

A. Changes in Protein Nitrogen and Activity of Pepsin Solutions at Various pH Exposed to Ultra-Violet Light

The results of an experiment in which pepsin solutions at various pH were exposed to light from a mercury arc are shown in Table II. As in the case of radium inactivation practically no denatured protein

TABLE II

Change in Activity and Protein Nitrogen in Pepsin Solutions of Various pH Exposed to Ultra-Violet Light

pH........	0			1.7			3.0			4.65		
Buffer.....	1.0 N hydrochloric acid			N/50 hydrochloric acid			N/65 acetic acid			N/50 4.65 acetate		
Time	$[P. U.]^{Hb}_{ml.}$	P N/ml.	$[P. U.]^{Hb}_{mg. P N}$	$[P. U.]^{Hb}_{ml.}$	P N/ml.	$[P. U.]^{Hb}_{mg. P N}$	$[P. U.]^{Hb}_{ml.}$	P N/ml.	$[P. U.]^{Hb}_{mg. P N}$	$[P. U.]^{Hb}_{ml.}$	P N/ml.	$[P. U.]^{Hb}_{mg. P N}$
hrs.		*mg.*			*mg.*			*mg.*			*mg.*	
0	0.019	0.098	0.20	0.020	0.098	0.21	0.020	0.098	0.22	0.020	0.098	0.21
2.55	0.014	0.076	0.18	0.015	0.070	0.215	0.017	0.080	0.21	0.017	0.084	0.20
5.00	0.0092	0.058	0.16	0.0095	0.050	0.19	0.0118	0.070	0.17	0.013	0.078	0.17
8.75	0.0054	0.030	0.18	0.0050	0.018	0.28	0.0077	0.058	0.13	0.0086	0.063	0.14

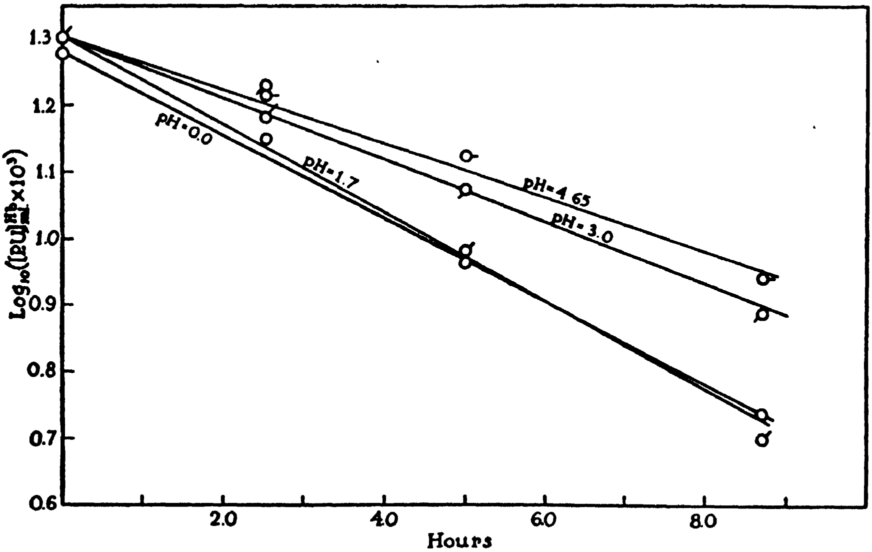

FIG. 1. Inactivation of pepsin by ultra-violet light at different pH

appears in the solution and the loss in activity is accompanied by a corresponding decrease in the total protein nitrogen of the solution; *i.e.*, the activity per mg. protein nitrogen remains constant throughout the experiment. The rate of inactivation depends upon the pH and is a maximum at about pH 2.0 and decreases as the pH becomes more alkaline. The pH corresponding to the maximum rate of inactivation agrees with that found by Collier and Wasteneys (5) and is slightly less acid than that reported by Pincussen and Vehara (3). The reaction follows approximately a monomolecular course, as shown in Fig. 1, in which the log of the activity is plotted against the time in hours.

Experimental Procedure

A solution of three times crystallized pepsin was prepared in N/20 pH 4.65 acetate. It contained 2.5 mg. protein nitrogen per ml. 1 ml. of this solution was diluted with 25 ml. of the buffer noted in the table and the pH determined. 25 ml. of the solution was placed in 1 cm. quartz test-tubes arranged in a semicircle around a General Electric "Lab-Arc" at a distance of 8.5 cm. from the arc. The lamp was operated on 110 volts A.C. and 1.8 amperes and was allowed to run 1 hour before the experiment was started. The activity and protein nitrogen were determined, as described for the radium experiments, except that with very dilute pepsin solutions it was necessary to use 5 ml. for the protein nitrogen determination. Control tubes containing 25 ml. of the solution in glass test-tubes were placed beside the quartz tubes. There was no change in activity or protein nitrogen in the solution in the glass tubes showing that the inactivation was due entirely to ultra-violet light. The temperature of the solutions was 15°C. The positions of the quartz tubes were interchanged at intervals of about 20 minutes so that any local variations in the light intensity were distributed. Special control experiments showed that the rate of inactivation was the same in the various tubes.

The analytical work was done by Mr. Nicholas Wuest.

SUMMARY

1. The loss in activity of crystalline pepsin solutions when exposed to beta and gamma rays from radium or to ultra-violet light is accompanied by a corresponding decrease in pepsin protein.

2. The rate of inactivation by ultra-violet light depends upon the pH and is a maximum at about pH 2.0.

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MOLECULAR WEIGHT, MOLECULAR VOLUME, AND HYDRATION OF PROTEINS IN SOLUTION

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The gram molecular weight and volume of a dissolved substance may be calculated from the osmotic pressure of the solution. Osmotic pressure is affected only slightly by hydration and so furnishes no precise information as to the size of the hydrated molecule as it exists in a solution. The radius of the hydrated molecule in solution, and hence the gram molecular volume of the hydrated solute, may be determined from diffusion measurements. The difference between this figure and the gram molecular volume, as found by osmotic pressure, therefore represents the amount of hydration. The hydration may also be calculated from viscosity measurements. These two independent methods for the estimation of hydration give essentially the same values for the hydration of crystalline hemoglobin and crystalline trypsin.

Molecular Weight from Osmotic Pressure

The gram molecular weight of a substance in solution may be defined as that quantity of dry substance which, when dissolved in 1 liter of solvent, gives rise to an osmotic pressure of 22.4 atmospheres at 0°C. If the osmotic pressure of a solution is known, therefore, its molar concentration may be calculated. Since there are 6.06×10^{23} molecules in a gram molecule the average weight of the individual molecules may be found if the weight concentration of the solution is also known. This figure represents the average dry weight of the individual molecules of solute for which the membrane is impermeable but furnishes no definite information as to their size. Solvation of the molecules increases their size but does not change the number of molecules and affects the osmotic pressure only by decreasing the quantity of free solvent. This decrease in the quantity of free solvent is not noticeable experimentally except in concentrated solutions or when the solvation is large.

Calculations of the molecular weight from osmotic pressure determinations involve the following assumptions:[1]

1. The system is at equilibrium.
2. The membrane is permeable to the solvent but impermeable to the solute in question.
3. The osmotic pressure is proportional to the concentration (van't Hoff's law).
4. The molecules of solute are all of the same size.

In the case of collodion membranes and aqueous solutions of proteins the first three conditions are fulfilled but the fourth may or may not be true. The protein molecules themselves may vary in size and in addition they may be combined with small ions or molecules which are thus prevented from free diffusion through the membrane, as in the Donnan equilibrium. In this case the osmotic pressure is due to both the protein molecules and the excess concentration of inorganic ions and the value calculated for the molecular weight represents the average of these various molecular species present. The complication due to Donnan equilibrium may be avoided experimentally by measurements made at the isoelectric point of the protein. The effect of neutral salts also furnishes a test for the presence of such Donnan pressures.

Radius of Molecules from Diffusion Measurements

The radius of the molecule determines the rate of diffusion in accordance with Einstein's equation (1)

$$D = \frac{RT}{N}\,\frac{1}{6\pi r\eta}$$

R = gas constant
T = absolute temperature
N = Avogadro's number 6.06×10^{23}
η = viscosity of solvent
r = radius of molecule
molecular volume = $4/3\ \pi\ r^3 N$

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[1] For a discussion of the osmotic pressure of hemoglobin solutions see Adair, G. S., *Proc. Roy. Soc. London, Series A*, 1928, **120**, 573.

Thus, if the diffusion coefficient of the solute is known the radius of the molecules and hence the gram molecular volume may be calculated. This value for the radius represents the radius of the particle which actually moves in the solution and therefore includes any solvent carried with the molecule. The following assumptions are involved in Einstein's equation:

1. The diffusing particles are few and large compared to the molecules of the solvent.
2. They are spherical.
3. They are electrically neutral.
4. They are impelled by a force equal to the osmotic pressure as given by van't Hoff's law against a resistance as given by Stokes' law.

As in the case of osmotic pressure the effect of ionization is the most important source of error with protein solutions. The presence of charged molecules may again be tested for by determining the effect of neutral salts and of the pH. If the molecules are not of the same size a constant value for the diffusion coefficient will not be obtained but the value will decrease as the experiment proceeds since the smaller particles will diffuse out faster. It is important, therefore, to continue the experiment until a large proportion of the solvent has diffused out; or better, to repeat the measurement on the first part of the diffusate, in order to be sure that the diffusion coefficient is actually the same for all of the solute. Otherwise entirely erroneous values may be obtained. The determination may be made conveniently and accurately as described by Northrop and Anson (2).

Calculation of Hydration from Osmotic Pressure and Diffusion Measurements

If the osmotic pressure and the diffusion coefficient of a solution are known, then the degree of hydration of the molecules of the solute can be determined as follows:

Let M be the gram molecular weight of the dissolved substance as determined from osmotic pressure measurements,
r the average radius of the molecules as determined from diffusion measurements,
S the specific volume of the dry substance,

then the gram molecular volume of hydrated molecules equals $4/3\ \pi r^3 N$ and the gram molecular volume of non-hydrated molecules equals $S\,M$.

Volume of water of hydration (if water is used as solvent) equals

$$4/3\ \pi r^3 N - S\,M$$

and

$$\frac{4/3\ \pi r^3 N - S\,M}{M}$$

equals volume of water of hydration per gram of dry solute, or

$$\frac{4/3\ \pi r^3 N - S\,M}{N}$$

equals volume of water of hydration per molecule solute.

Determination of Hydration from Viscosity Measurements

An independent method for the determination of the amount of hydration of substance in solution is the measurement of viscosity (3). This method applies to the case of molecules or particles large as compared with the size of the molecules of the solvent and consists in determining the relative viscosity of the solution as compared with the viscosity of the solvent. The volume of the solute may be calculated by aid of the empirical formula

$$\eta = \frac{1 + 0.5\,\phi}{(1 - \phi)^4}$$

where η equals the relative viscosity of solution and ϕ equals the volume of solute expressed as the fraction of the total volume of the solution. The formula was found to hold well for a large number of solutions or dispersions of molecules of relatively large size.

The two methods of determining the degree of hydration were used here in the case of such substances as hemoglobin and crystalline trypsin, and the results show that there is quite a close agreement between the two methods.

The results are summarized in the following table:

	Molecular weight	Average radius of hydrated molecule	Water of hydration per gm. dry wt.	
			Osmotic pressure diffusion method	Viscosity method
			$cm.^3$	$cm.^3$
Hemoglobin	67,000	2.73* $\times 10^{-7}$	0 to 0.14	0.13†
Isoelectric gelatin	61,500	(5.4 $\times 10^{-7}$)	(5.8)	5.9
Crystalline trypsin	35,000	2.6 $\times 10^{-7}$	0.54	0.49

Technique.—Osmotic pressure, Northrop and Kunitz (4), Adair (5); diffusion, Northrop and Anson (2).

[*] Svedberg, T. (Colloid chemistry, American Chemical Society Monographs, New York, The Chemical Catalog Co., 2nd edition, 1928, 165) obtains a value of 0.0342 cm.^{2}/day corresponding to a molecular radius of 3.35×10^{-7} cm. Svedberg's measurements were made while the molecules were moving under the influence of centrifugal force and the difference in the value may be due to the fact that the molecules are not spherical. In this case they might be oriented in a gravitational field and would, therefore, move through the liquid at a rate different from that determined by diffusion alone.

[†] Adair and Robinson (*J. Physiol.*, 1931, **72**, 28) obtained a value of 0.2 ml. water per gm. hemoglobin from measurements of the water absorbed by the dry protein from ammonia sulfate solutions.

EXPERIMENTAL

Hemoglobin

Osmotic Pressure.—Measurements of Adair (5).

Diffusion.—Northrop and Anson (2).

Viscosity.—Measurements were made of the viscosity at 5°C. of various concentrations of CO-hemoglobin in M/20 phosphate buffer pH 6.8 using an Ostwald viscosimeter; specific volume of dry hemoglobin equals 0.75.

The data given in Table I show that the hydration of hemoglobin under the conditions of the experiment decreases with the dilution and is about 0.1 ml. per gm. of hemoglobin at concentrations below 2 per cent. The diffusion experiments of Northrop and Anson were done under the same conditions of hemoglobin in the range of 1–2.5 per cent. The experiments of Northrop and Anson show that at 5°C. the diffusion coefficient for 2.5 per cent hemoglobin in N/20 phosphate buffer pH 6.8 is between 0.0434 and 0.0401 cm.^{2}/day. The calculated radius of the molecules is between 2.65×10^{-7} and 2.86×10^{-7}. Hence $4/3\pi r^3 N$ equals between 47,300 cm.^{3} and 59,500 cm.^{3} From osmotic pressure measurements (Adair) $S\ M = 67{,}000 \times 0.75 = 50{,}000$.

Volume of water of hydration per mole = between 0 and 9,500 cm.^{3}
" " " " " " gram = " 0 " 0.14 cm.^{3}

Thus it is seen that in the case of hemoglobin the amount of water of hydration per gram of protein, as obtained by viscosity measurements, is so small as to be within the experimental error of the diffusion measurements.

Crystalline Trypsin

Osmotic Pressure Measurements.—Northrop and Kunitz (6).

Diffusion Measurements.—Scherp (7).

Viscosity.—Viscosity measurements of solutions of crystalline trypsin were made under conditions similar to those employed in the determination of the molecular weight of crystalline trypsin by means of osmotic pressure measurements, as well as in the determination of the diffusion coefficient of crystalline trypsin as carried out by Dr. Scherp in this laboratory.

TABLE I

Viscosity Measurements of CO-Hemoglobin, pH 6.8 at 5°C.

Concentration of protein	Relative density at 5°C.	Relative viscosity*	Calculated volume of solute in cm.³/100 cm.³ solution	Specific volume per gm. protein	Volume of water of hydration per gm. hemoglobin
gm./100 ml. solution				*cm.³*	*cm.³*
2.10	1.006	1.084	1.85	0.88	0.13
4.20	1.012	1.175	3.65	0.87	0.12
6.30	1.018	1.290	5.60	0.89	0.14
8.36	1.024	1.445	7.90	0.95	0.20
10.45	1.030	1.610	10.15	0.97	0.22

* These values are much lower than those reported by Lewis and Loughlin (*Biochem. J.*, London, 1932, **26**, 480) and give rise to correspondingly lower values for the hydration. This difference is not due to the salt present since repetition of the measurements with salt-free hemoglobin solution gave practically the same figures for the viscosity of the solution as found for hemoglobin solution in M/2 phosphate buffer.

The procedure was as follows. A solution of crystalline trypsin in M/10 acetate buffer pH 4.0 was made salt-free by dialysis in the cold room against N/10,000 hydrochloric acid. The dialyzed trypsin was then diluted with equal volume of saturated magnesium sulfate in M/10 acetate buffer pH 4.0 and dialyzed against a definite volume of trypsin-free 0.5 saturated magnesium sulfate in M/10 acetate buffer pH 4.0 until equilibrium was established as indicated by the reading of a manometer tube inserted in the collodion bag containing the trypsin solution. The outside solution was found to be free of any trypsin. A series of dilutions was then made of the trypsin solution by means of the outside magnesium sulfate solution and viscosity measurements were made at 5°C. The results are shown in Table II. The specific volume of dry trypsin was taken as 0.75 ml./gm. which was found to be common for proteins of the albumin type. The average value of the water of hydration of crystalline trypsin when dissolved in 0.5 saturated magnesium sulfate pH 4.0 was thus found by the viscosity measurements to be 0.5 ml. per gm. dry protein.

TABLE II

Viscosity at 5°C. of Various Concentrations of Crystalline Trypsin in 0.5 Saturated Magnesium Sulfate and M/10 Acetate Buffer pH 4.0

Concentration of trypsin	Time of outflow	Relative viscosity	Calculated volume of hydrated trypsin in cm.^{3}/100 cm.^{3} solution	Volume of hydrated trypsin per gm. dry trypsin	Water of hydration per gm. dry trypsin
gm./100 ml.	*sec.*			*cm.^{3}*	*cm.^{3}*
0	203.4	1.000	0		
0.8	212.6	1.045	1.00	1.25	0.50
1.6	221.5	1.089	2.00	1.25	0.50
2.4	231.0	1.135	2.90	1.21	0.44
3.2	241.4	1.187	3.90	1.22	0.47
4.0	257.0	1.265	5.15	1.29	0.54
Average					0.49

The radius of hydrated trypsin molecules under the same conditions, as determined by Scherp from diffusion measurements, was found to be 2.6 × 10^{-7} cm. The volume of one mole of hydrated trypsin is therefore

$$(2.6 \times 10^{-7})^3 \times 4/3\ \pi \times 6.06 \times 10^{23} = 44{,}700 \text{ cm.}^3$$

The molecular weight of the trypsin in solution under the same conditions was found by osmotic pressure measurements to be about 35,000 gm. The molecular volume of the non-hydrated trypsin equals 26,000 cm.^{3} Hence, water of hydration per mole of trypsin equals 19,000 cm.^{3} Water of hydration per gram dry trypsin equals 19,000/35,000 equals 0.54 cm.^{3}/gm. Thus, the value for hydration of trypsin, as determined by diffusion experiments in connection with osmotic pressure measurements checks quite closely with the value obtained by viscosity measurements. This agreement serves as a check for the viscosity formula and justified the application of Einstein's diffusion formula to protein solutions.

Gelatin

The hydration of gelatin, as calculated from osmotic pressure and from viscosity measurements, has been described in a previous paper (8). The value of the hydration so obtained was 6 cm.^{3} water per gram dry gelatin in 3 to 5 per cent solutions.

Diffusion measurements were made with gelatin solutions in order to see whether the hydration, as determined by this method in connection with the osmotic pressure measurements, agrees with that calculated from viscosity. If a 5 per cent solution of gelatin pH 4.7 in M/1000 acetate buffer was allowed to diffuse, a constant value for the diffusion coefficient of 0.05 cm.^{2}/day was obtained. However, if the first diffusate was replaced in the cell and the experiment repeated, a much larger value for the diffusion coefficient was found. Gelatin solutions, therefore, as was to be expected, are not homogeneous but the relative size of the particles or their relative amount, do not differ sufficiently to cause a noticeable drift in the diffusion coefficient as determined from any one experiment. Trial calculations show that a mixture containing 30 per cent of particles of radius 2 and 70 per cent of particles of radius 1 will diffuse in such a way as to give a value for the diffusion coefficient, as calculated from the total amount diffused, which does not vary over 10 per cent until more than 75 per cent of the total original quantity has diffused out. Such a mixture, however, would give an entirely different value for the diffusion coefficient if the measurements were repeated on the diffusate. This is the result obtained with the gelatin. The results are complicated in addition by the fact that some hydrolysis of the gelatin occurs during the experiment.

Since the value of the diffusion coefficient has no physical significance unless the diffusing particles are of nearly uniform size, the results with gelatin are of doubtful significance.

SUMMARY

1. The gram molecular weight of a substance may be calculated from the osmotic pressure of its solution.

2. The radius of the hydrated molecule and, hence, the gram molecular volume of the hydrated solute may be determined from diffusion measurements. The hydration of the molecules may, therefore, be calculated from osmotic pressure and diffusion measurements.

3. Hydration may also be determined by viscosity measurements. Hydration of crystalline hemoglobin, crystalline trypsin, and gelatin have been determined by these methods and found to be as follows:

	Molecular weight	Average radius of hydrated molecule	Water of hydration per gm. dry wt.	
			Osmotic pressure diffusion method	Viscosity method
			cm.^{3}	*cm.*^{3}
Hemoglobin	67,000	2.73×10^{-7}	0 to 0.14	0.13
Isoelectric gelatin	61,500	(5.4×10^{-7})	(5.8)	5.9
Crystalline trypsin	35,000	2.6×10^{-7}	0.54	0.49

The results with gelatin calculated from the diffusion measurements are uncertain since gelatin solutions are not homogeneous.

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A NOTE ON THE RESPIRATION OF ARBACIA EGGS*

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Tang (2), with Gerard (4), measured the respiration of resting and fertilized eggs of *Arbacia punctulata* incidental to a study of the influence of oxygen tension on respiration. Absolute values in cubic millimeters of oxygen per million eggs per hour were reported. For one season the average for unfertilized eggs was 33.6 at 24.7°C., and a few runs with fertilized eggs gave figures five times as high. Next season the average for freshly fertilized eggs was 118 at 25°C., and a few experiments with unfertilized ones gave again one-fifth the respiration, or 23.5. The eggs of the first season, however, averaged 77 micra in diameter and possessed, therefore, almost 25 per cent more volume than those of the second season, with a diameter of 72 micra on the average. Expressed per unit volume of eggs, therefore, the data for the two seasons' experiments are less than 15 per cent apart.

Whitaker (5) has measured again the respiration of this egg and obtained absolute values per unit volume only one-third to one-half as great as those we reported. His results fitted data for the respiration of other related or unrelated eggs, obtained by himself and others, better than did ours, and our procedures and findings were adversely criticized (6). It seemed desirable to clear up, if possible, the discrepancy and establish the correct value; the more so since this datum is becoming of some theoretical importance for recent respiration studies (*e.g.* (1) and (6)).

We have therefore repeated the measurements with careful control of all points of difference in the conditions of our respective experiments, though most of these had been previously considered. Since

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all agree that fertilization gives about a fivefold increase in respiration, and the findings on resting eggs have been especially questioned, the present experiments have been limited to the latter.

There are four major differences to be considered: preparation of the egg suspension; temperature used; conditions during the run in the manometers; measurement of amount of material used.

Our preparation involved: opening the female urchins, placing the ovaries in sea water, straining the thick suspension of eggs through cheese-cloth, and using as such or after two washings with sea water by gentle hand centrifuging. Whitaker allowed eggs to shed through the gonopores of inverted half shells and washed by decantation only. He believed our eggs suffered cortical damage during preparation, with consequent abnormally high respiration. In the present work, we performed two series of tests in which alternate urchins in succession were handled by one or the other method, the two groups collected, and respiration measured. There was no difference. Dr. A. J. Goldforb has kindly permitted us to mention unpublished experiments of similar import. Fertilization, cytological changes, development, and permeability of eggs shed by one female through the gonopores was the same as that of eggs shed from an isolated ovary of the same animal. Likewise he found filtration through cheese-cloth without effect.[1] We have none the less used Whitaker's procedure in the present work. In a few cases, only the Aristotle's lantern was removed and the eggs allowed to shed with a minimum of contamination by body fluids or débris. Further, filtered sea water was used for collecting and washing the shed eggs.

Our experiments were performed at 24.7° or 25°C., Whitaker's at 21°C. For comparing values he corrected our results to 21°, assuming a temperature coefficient of 2.0. As reported in a paper soon to be published, this value is approximately correct only for fertilized eggs, that of resting eggs is over 4. The temperature correction Whitaker applied was, therefore, too low. We shall return to this later.

The most serious discrepancy, Whitaker believed, resulted from the treatment in the manometers for measuring oxygen consumption. Since the chambers containing the respiring egg suspension have liquid and gas phases and oxygen diffusion is slow, continued agitation is needed to maintain the oxygen content of the liquid. This is achieved by a rhythmic to-and-fro motion of the manometer and, if inadequate, the measured respiration will be less than the true value. The other danger is that this shaking, if too vigorous, will damage the delicate egg cortex, even to the extent of cytolysis, and so produce artificially high oxygen consumptions. It is into this latter error, Whitaker concludes, that we fell. Though our actual shaking conditions were specified only for the fertilized eggs, the questions of oxygen penetration and egg injury were fully considered. Our fluid layer was less than 2 mm. deep, Whitaker's 3.5 or 7, and the required agitation is, of course, much less with thinner layers. In fact, in the unfertilized egg experiments, shaking rates varied from 40 to 60 per minute over arcs of 5 to 10 cm. with no regular differences in results. After a run, the eggs were fertilized and regularly showed over 90 per cent normal cleavage. The rates on fertilized controls were also increased five times, which is difficult to reconcile with an initially high respiration of the unfertilized eggs due to injury. In the present work we have used even less shaking

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[1] Goldforb has also observed a progressive increase in the respiration of unfertilized eggs on standing, beginning some 5 hours after shedding. Our runs have mostly terminated about this time, but we have noted in many longer runs a slight tendency to increased respiration at the end. We would also like to note, for later comparison, that he was not able to obtain constant volume determinations by centrifuging, nor were we.

TABLE I

1 cc. unfertilized *Arbacia* eggs (477,000). 0.2 cc. N NaOH in inset. 21°C. Start = 94 per cent fertilization, end = 95 per cent fertilization. Manometer constants all about 0.8.

Shaking	Time	Change in level			
		1	2	3	4
	min.	*mm.*	*mm.*	*mm.*	*mm.*
48/min., 5.5 cm. arc	0–20	2.3	2.7	2.5	2.7
	20–40	2.2	2.2	2.2	2.1
	40–60	2.6	2.3	2.3	2.8
Q_{O_2} 1 hr.		**12.1**	**12.8**	**13.6**	**13.0**
	60–120	7.6	7.6	6.9	7.7
Q_{O_2} 2 hrs.		**12.7**	**13.0**	**13.3**	**13.2**
72/min., 5.5 cm.	120–150	4.1	3.6	3.5	3.6
	150–180	4.2	4.0	4.0	4.4
Q_{O_2} 1 hr.		**14.2**	**13.4**	**14.6**	**13.8**
	180–300	14.9	14.2	13.3	14.3
Q_{O_2} 3 hrs.		**14.3**	**14.0**	**14.7**	**14.0**

than recommended by Whitaker (50 per minute, 5.5 cm. arc), except for deliberate tests at higher rates. The protocol of such an experiment is given in Table I, and shows a minimal effect (not progressive with duration) with the fastest speed our shaker attains. Also, after several hours of fast shaking the same high fertilization (95 per cent) was obtained as at the start.

The measurement of egg quantity used is not a simple matter. Whitaker centrifuged his suspensions to constant egg volume in vaccine tubes (15 minutes, force not given) and, though realizing the possibility of errors due to imperfect packing, assumed these were small. He mentions no attempt to check this point. We counted the eggs in our suspensions with a hemocytometer and measured diameters with an ocular micrometer, from which data egg volumes per cubic centimeter of suspension are easily obtained. (In recalculating our data, Whitaker overlooked the given diameters and assumed a constant one of 74 micra which resulted in considerable discrepancies.) We had previously found centrifugation unsatisfactory in rough tests; now we have carefully compared both methods.

The measurement of egg diameters presents little difficulty. In good batches the majority of cells appear round and vary less than 10 per cent in individual measurements. Two observers, measuring different egg samples from the same suspension, regularly checked within 1.5 per cent. A typical result on fifteen eggs was: R.W.G.: 74.2 micra ± 0.3; B.B.R.: 73.8 ± 0.4. To the extent that eggs are slightly oblate and tend to settle on the flatter side, the egg volume calculated from the measured diameter will be high, and the rate of respiration calculated per unit volume correspondingly too low. This factor could hardly amount to 10 per cent and, if present, would make even our values below the correct ones.

Enumeration of the eggs is more difficult. Ideally a large counting chamber, holding up to a cubic centimeter of suspension, should give the best results. Actually, we have not found dilution followed by counting eggs in 0.5 cc. on a mechanical stage to be satisfactory. The ordinary blood-counting chamber, with 0.1 mm. between slide and cover-slip, permits counting the eggs in 0.9 c.mm. of undiluted suspension. The danger is that the large eggs settle so rapidly that all contained in a drop much thicker than 0.1 mm. may reach the slide surface before the cover-slip is placed and the fluid thinned. This does occur if the preparation is not made rapidly, and abnormally high (up to 30–50 per cent) counts result. The egg distribution per square is then likely to be very irregular. The further danger also exists that such a small sample is not fairly representative of the suspension. We have adopted a procedure of rapidly placing a drop, at once sliding on the cover-slip, and counting the four large corner squares (often the four side ones as well). Each observer counted five successive drops, the suspension being adequately mixed each time, and an average value taken. With practise, quite consistent results are obtained. The raw data of one run are given as an example (Table II).

The method has been further checked by counting a known suspension, diluting two and five times, and recounting. Results of such a test were: original suspension = 300,000/1 cc.; diluted two times = 295,000/2 cc.; diluted five times = 320,000/5 cc.

In six experiments, after obtaining the size and number of eggs in the suspension and calculating the volume of eggs per cubic centimeter, volume was directly measured on the centrifuge. Graduated centrifuge tubes were used and, though less accurate than vaccine tubes, agreed well in duplicate and certainly gave approximately correct values. 2 to 5 cc. of suspension were used and spun for 1 to 10 minutes by hand or electric centrifuge. Although absolute constancy of volume was rarely attained, an approximate constancy was reached in 3 to 5 minutes. A volume that had become constant on hand centrifuging (radius 16 cm., 25 R.P.S.; centrifugal force = 400 × gravity) would shrink somewhat in the electric instrument under greater force (radius 18 cm., 32 R.P.S., C = 750 g.).

TABLE II

Observer	Drop	Cells per 1 × 1 × 0.1 mm. corner square				Average	Mean and *P.E.*
B.B.R.	1	28	29	32	31	30.0	
	2	30	26	36	33	31.1	
	3	27	31	21	31	27.5	
	4	30	31	32	30	30.8	
	5	26	27	33	30	29.0	29.7 ± 0.5
R.W.G.	1	36	37	35	30	34.5	
	2	35	34	34	26	32.2	
	3	31	31	34	30	31.5	
	4	25	34	27	30	29.0	
	5	29	27	35	30	30.1	31.5 ± 0.6

The ratio of minimum centrifuged volume to calculated one varied between 1.7 and 2.0. (In one case not included a ratio of 3.0 was obtained, but centrifugation was probably incomplete.) The average ratio was 1.8. That is: assuming the eggs were correctly counted and measured, the volume as determined by centrifuging was 80 per cent too high, and respiration rates calculated on such a basis correspondingly low.

As an extra check on the centrifuge technique we used the Harvey centrifuge-microscope, kindly made available to us by Dr. E. N. Harvey. Eggs prepared as usual and the same after straining through two layers of 60 micra bolting-cloth to remove the jelly were used. At the slowest speed obtainable (higher than that usually used, C = 550 g.) the unfiltered eggs settled into a very loose mass. Individual eggs were rarely in contact with neighbors and often an egg radius separated them. This was largely due to the jelly, since the filtered eggs did come in contact and became deformed. Even here, however, many and large spaces were clearly visible at the edges of faceted surfaces. With still higher speeds (C = 1050 g., individual eggs were well stratified) the same pictures remained, though some further packing occurred. The conclusion seems clear that respirations calculated per unit volume, determined by the centrifuge, are subject to a large and variable error.

It will require further tests to determine the volume error for other eggs and cells. It is possible though unlikely (because of varying jelly masses, sizes, and toughness of membranes) that a uniform correction will apply to all of Whitaker's data, in which case the relation he has pointed out, that various eggs tend to the same respiratory rate after fertilization, will remain valid. Pending such controls, this must be regarded as uncertain; and the absolute values must surely be revised. It may be mentioned that data of other workers on different species of urchin eggs, which agree with Whitaker's values for *Arbacia*, were likewise based upon egg volumes determined on the centrifuge.

It remains to report the actual respiration rates obtained in the present experiments, carried out almost entirely according to Whitaker's procedure except for the measurement of egg volume. The Q_{O_2} per million eggs, in eleven experiments with three to six runs each, varied from 11.3 to 33.3. This is the same variability from batch to batch previously encountered. The average value at 21°C. was 19.5. In terms of the unit Whitaker has used, cubic millimeters O_2 per hour per 10 c.mm. of eggs, this becomes, for these eggs, 0.9. (Second decimals are not significant.) Whitaker obtained, also at 21°C., 0.4–0.5 for the unfertilized eggs. If this be corrected for the volume error of centrifuging (times 1.8) it becomes 0.7–0.9, in essential agreement with the above. Tang's result for unfertilized eggs at 24.7°C., in these units, is 1.4; Tang and Gerard's for fertilized eggs at 25°C., 6.1 (1/5 = 1.2 for the unfertilized equivalent). Applying a temperature coefficient of 4.0 to the unfertilized eggs, 2.0 to the fertilized (3), these values become, at 21°C., respectively 0.8 and (1/5 the fertilized value) 1.0. It is clear that all experimental series are in fair agreement when correct egg volumes are obtained and proper allowance made for temperature differences.

SUMMARY

Methods used in preparing *Arbacia* eggs for respiration studies, in carrying through the manometric determinations, and in estimating egg quantities have been reexamined.

Discrepancies in previous results are almost entirely due to a steady error in measuring egg volume by centrifuging. Volumes so obtained averaged 80 per cent too high.

The respiration of unfertilized eggs of *Arbacia punctulata* at 21°C. is 0.9 c.mm. O_2 per hour per 10 c.mm. of eggs.

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A PHOTOELECTRIC DENSITOMETER FOR USE WITH SUSPENSIONS

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I

In studies of respiration and fermentation it is necessary to measure the population density of cell suspensions. Laborious and relatively inaccurate methods in common use are a serious handicap in the time consumed, and they often limit the delicacy of experiments requiring their use.

A device here described was constructed to give a simple and sensitive method of preparing yeast suspensions of known concentration for studies using Warburg respirometers; with suitable precautions, it should be useful in determining growth curves of microorganisms. Peskett (1927) used nephelometry for measuring the growth of yeast (using $BaSO_4$ standards); Williams, McAlister, and Roehm (1929) constructed a special thermocouple for measuring indirectly the light absorbed by various suspensions of microorganisms. Richards and Jahn (1933) constructed two nephelometers each using a source of light and a single Weston Model 594 photoelectric cell arranged in such a way that in one the whole suspension and in the other a small part is placed in the path of the light. On careful examination, none of the devices previously described was found accurate to less than 3 per cent (*cf.* Richards (1932) for comparison of the accuracy of hemocytometer, hematocrit, and earlier nephelometers). The extreme sensitivity of photoelectric cells necessitates certain precautions which have often been neglected in the construction and use of photoelectric densitometers. We shall describe the precautions which must be taken with the instrument and with the suspension of the microorganism in order that readings accurate and reproducible to within 1 per cent may be obtained.

II

The circuit and a schematic diagram of the apparatus are given in Fig. 1. A single, high intensity light-source (100 watt projection bulb) is directed at two longitudinal slits 35 mm. × 8 mm. in a block receptacle holding a 20 × 200 mm. Pyrex milk culture tube in a rigid holder, behind which are two Visitron photoelectric cells, Type 71 A.V. The currents flowing from the two cells are balanced in an ordinary Wheatstone bridge, Leeds and Northrup Type S, using a box galvanometer, Leeds and Northrup Catalog 2420 B. Weston Photronic cells were found not to be as stable as vacuum photoelectric cells.

If a relatively delicate instrument is desired it is essential to use two photoelectric cells and to balance the currents from each by the method indicated.[1] This insures almost complete independence of fluctuations in the voltage applying to the light-source, a significant variable in most laboratories; the light intensity varies almost as the square of the voltage variation. An apparatus containing a single photocell requires either continuous adjustment of the line voltage or some

FIG. 1. *A*. Diagram of the circuit used. For detailed specifications as to the apparatus used see text.

Explanation of symbols:

i_1 = current from Photocell I.
i_2 = current from Photocell II.
R_1 = fixed resistance.
R_2 = variable resistance.
P_1 = Photocell I.
P_2 = Photocell II.

The intensity of light falling on Photocell I is referred to in the text as I_1, and on Photocell II as I_2. The galvanometer may be protected from damage by short circuiting etc., by placing 1,000 ohms fixed resistance in *CD* if desired.

B. Sectional side elevation of the apparatus. ① refers to the colloidal gold heat screen, ② to asbestos sheeting lining the lamp housing and shield, ③ to the special triangular brass holder mentioned in the text, and ④ to Photocell II.

C. The apparatus from above.

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[1] This scheme was used by Goos and Koch (1927) and by Partridge (1930); it is also employed in the Exton Scopometer of Bausch and Lomb Optical Co., in the APC Turbidity Meter of Eimer and Amend, and in others. Most of the photoelectric densitometers obtainable from manufacturers are unsuitable for work with pure line strains of microorganisms because the suspensions must be transferred to special absorption cells for measurement, thus increasing the chances of infection.

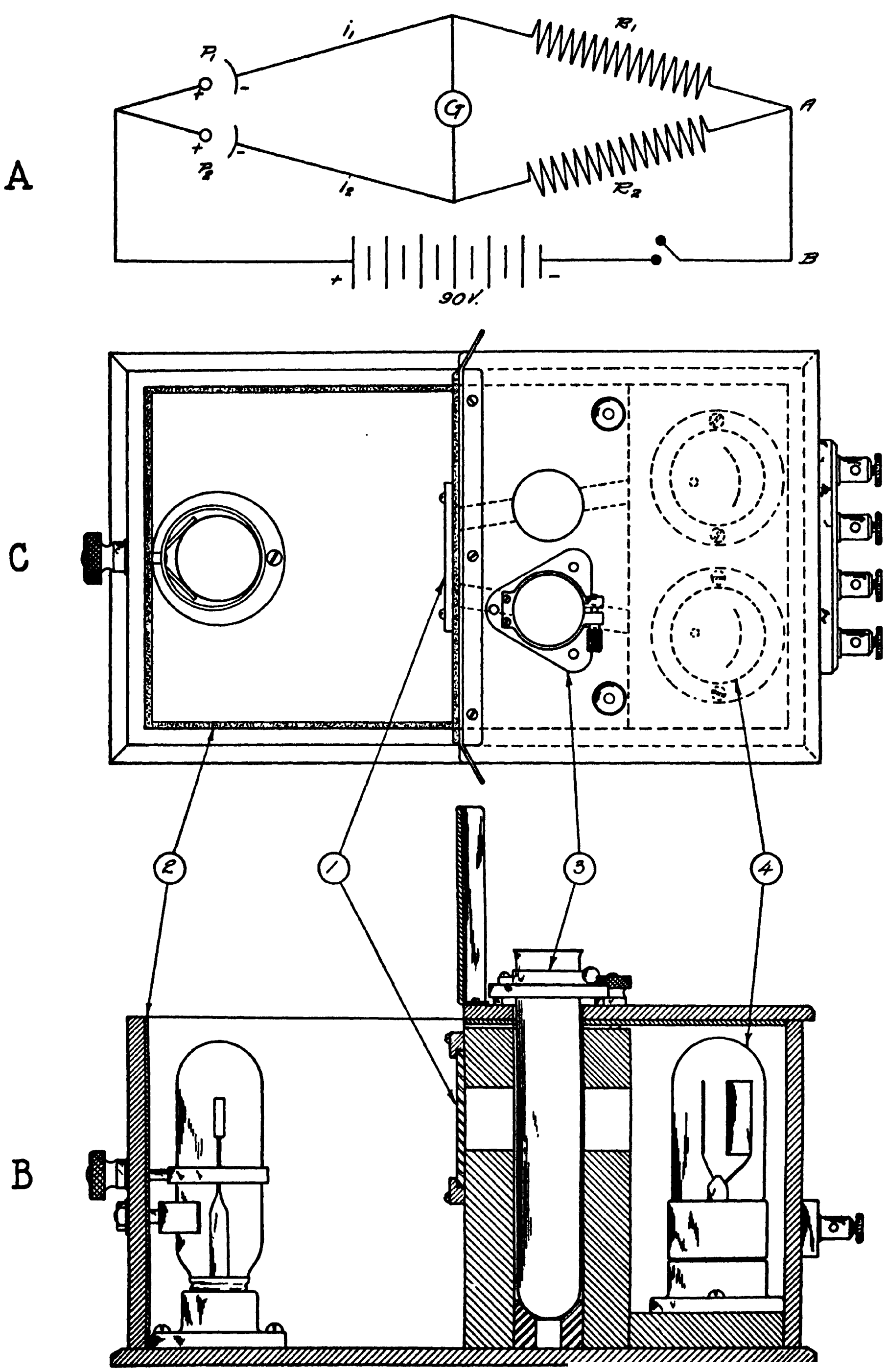

FIG. 1

device for automatic regulation. The former method is a source of considerable error in measurements, and the latter is more expensive and less satisfactory than the present arrangement. By test with our apparatus, the line voltage could fluctuate as much as 16 to 20 per cent with only 0.8 to 1.0 per cent change in the readings made.

The angular relation of the photocell-sensitive surface to the concentrated filament must not vary, otherwise the calibrations must be repeated. The radius of curvature of Pyrex test-tubes is not constant, and hence the unequal reflection at different points would alter the amount of light reaching the photocell if the culture tube were turned about its axis. The first difficulty was overcome by using a spring holder near the top of the lamp, a rigid base for the lamp, and rigid construction of the box; the second by the use of a screw-holder mounting for the tube with a three point scalene triangle base. It was necessary to make separate dilution curves for several tubes on the same standard suspension of yeast in order to determine empirically the correction factors for differences in radius of curvature of each tube.

The lamp is left open to the air to facilitate cooling, but the culture tube, photocells, etc. are protected from reflections and dust as shown in the diagram, Fig. 1, *B* and *C*. All inside surfaces were blackened with India ink. It was found advisable to protect the suspensions from the heat of the lamp by inserting a colloidal gold filter, as in Fig. 1, *B*, and by having a cool air stream from a small electric hair dryer directed into the lamp house. It is necessary to have a light-source of high intensity to yield a high current from the photocell. Unfortunately much infra-red radiation accompanies any convenient light-source. The precautions taken eliminated nearly all the heat, so that the temperature of a given suspension increased only 1°C. in 10 minutes. Since it takes only 2 to 3 minutes for a determination of the density of a suspension, this factor is insignificant.

In Fig. 1 the resistances R_1 (the fixed resistance) and R_2 (the variable resistance) may be related as $\frac{R_2}{R_1} = r$. The currents i_1 and i_2 of the photocells are represented by

$$i_1 = k_1 I_1$$

and

$$i_2 = k_2 I_2.$$

At balance

$$i_1 R_1 = i_2 R_2,$$

or

$$r = \frac{R_2}{R_1} = \frac{i_1}{i_2} = \frac{k_1 I_1}{k_2 I_2} = \frac{k_1}{k_2} x,$$

where

$$x = \frac{I_1}{I_2}.$$

This means that the resistances R_2 and R_1 are measures of the intensities I_1 and I_2, varying only as $\frac{k_1}{k_2}$ varies. It is known that the quantities k_1 and k_2, characteristics of each photocell, vary with the temperature, with light intensity, and with the history of the cell. By making readings in the form of the ratio

$$\frac{R_2 \text{ for suspension}}{R_2 \text{ for suspending medium}}$$

where R_2 represents the bridge reading in ohms (at balance) for the variable resistances in each case, they become independent of such variations. Since R_1 is kept constant at 1000 ohms, R_2 may be used in place of r in this ratio, making it possible to calculate directly from the bridge resistance-settings. The reading for the suspending medium preceded and followed each set of three to four readings taken on a given suspension.

III

Dilution curves were made with yeast suspension (in 3.7 per cent dextrose solution), and with an ammoniacal India ink solution. When the ratio $\frac{R_2 \text{ for suspension}}{R_2 \text{ for suspending medium}}$ was plotted against the concentration of the sample, in per cent of the most concentrated sample, the points fell on an exponential curve. Put into a semi-log plot, this gave the straight lines shown in Fig. 2. The values for the yeast suspension shown in Fig. 2, were obtained on fresh pressed yeast (*Saccharomyces cerevisiae*), centrifuged and washed twice with 3.7 per cent dextrose solution, and then suspended in 3.7 per cent dextrose. (Pure strain cultures will be used in future experiments.) The concentration range over which the plot is rectilinear is wider than is likely to be required for most respiration studies; in this experiment 79.9 per cent = 3,780,000 cells/mm.^{3}, 10 per cent = 573,500 cells/mm.^{3}. Clumped cells were removed by drawing the suspended cells through a sterile Jena sintered glass filter (20 to 30 microns mesh).

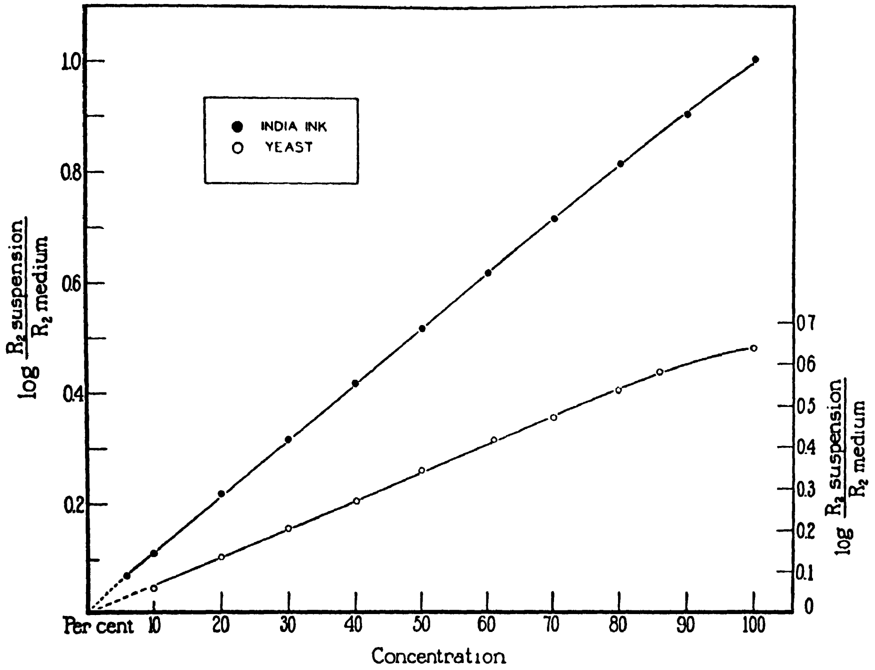

FIG. 2. Dilution curves, showing the exponential relation between the concentration of any given suspension and the ratio $\frac{R_2 \text{ for suspension}}{R_2 \text{ for suspending medium}}$ (see text) The log of the ratio is plotted against the concentration in per cent of the most concentrated sample, Line *A* refers to ammoniacal India ink suspensions; Line *B* to suspensions of pressed yeast in 3.7 per cent dextrose solution. The yeast cells were centrifuged and washed twice with the medium used. In making the dilutions of the yeast suspension 50 ml. of the heavy stock suspension were measured into separate flasks with a calibrated volumetric flask and the proper amount of medium added from a calibrated burette. Two burettes were employed in making the ink dilutions.

Each point represents the average of three determinations made on the same sample within about 3 minutes (see text for discussion of the accuracy of the instrument over a series of observations on the same sample). For yeast the density of each dilution was corrected for the increase in the number of cells from zero time; *i.e.*, from the moment when the density of the stock suspension was first determined. The rate of increase in opacity due to growth was determined at constant temperature and a correction for this increase was applied to the measurements of each dilution.

In using this instrument for determining rates of growth of yeast it is important to consider the effect of changes in the optical properties of the yeast cell upon measurements of density by an optical method. Richards (1932) has already shown that beyond the initial 24 hour period of growth (at 28°C.) the optical properties of the yeast cell change markedly. Also, when it is desired to prepare suspensions of known concentration from pure strain cultures it is necessary to use cultures of the same age, grown under the same culture conditions, as those used in constructing a dilution curve for the strain in question.

A dilution curve was also determined for a 1 week old culture of *Chlorella pyrenoidosa*. The curve is essentially the same as that obtained for yeast suspensions (*cf.* Fig. 2). It must be remembered, however, that unless the chlorophyl content per unit volume of cells is the same in different suspensions no comparable measurements of density will be obtained. (All the measurements were made at room temperature 22° ± 1.0°C.)

The empirical relation found agrees approximately with Beer's law, which states that the effect of concentration on the amount of light absorbed by a given non-colloidal suspension is: $I = I_0e^{-cd}$, where I = intensity transmitted, I_0 = intensity entering medium, c = concentration in grams per liter, d = thickness in cm., and e = the Napierian base. Evidence of the expected deviations from this "law;" *viz.*, at the upper and lower concentrations of the suspension (*cf.* Sheppard (1914)) can be seen in Fig. 2.

The sensitivity of the instrument makes standardization of technique essential. A given suspension will give progressively changing readings over a short period of time in the apparatus (quite apart from the factors already mentioned), due to the gradual settling of the suspended material. The suspension may be agitated continuously, without admitting air bubbles, by a syringe whose plunger is driven up and down by a small electric motor or, if simplicity is desired, by stirring with a glass rod at a uniform rate, for a constant, short time before making readings. For example, the suspension may be stirred by hand for 15 seconds at sufficient speed to get all the cells in motion, and the reading taken exactly 1 minute after cessation of the stirring in each case. The light-source was switched on the moment stirring ceased and used only during the period of observation, to prevent undue rise in temperature.

IV

The instrument used in the manner described is relatively more accurate than other methods of measuring population density and is considerably faster in operation.

The error in setting R_2, and hence in the ratio r, for any given single observation is limited by the error of the eye in detecting whether the galvanometer mirror deflects, and by the sensitivity of the instruments used. The per cent error in this setting may be calculated; for the instruments used it was found to be less than 1 per cent. This may be shown as follows:

Let

Δi = smallest current detectable with the galvanometer used in our set-up,

and

Δr = smallest change in r dependent thereon,

where

$$r \text{ is proportional to } \frac{i_1}{i_2} \text{ (\textit{cf.} p. 386)}$$

Then, using i_1, i_2, and r in the same sense as in Section II,

$$\Delta r = \frac{i_1 + \Delta i}{i_2 - \Delta i} - \frac{i_1}{i_2} = \frac{i_2\Delta i + i_1\Delta i}{i_2(i_2 - \Delta i)}$$

$$\Delta r = \frac{\Delta i(i_2 + i_1)}{i_2(i_2 - \Delta i)} = \text{approximately } \frac{\Delta i(1 + r)}{i_2}$$

or per cent error of r =

$$\frac{100\Delta r}{r} = \frac{\Delta i\left(1 + \frac{1}{r}\right)}{i_2} \times 100 = \text{approximately } 100\,\frac{2\Delta i}{i_2}$$

The magnitude of this quantity was found by substituting the values found. On the galvanometer used 1 mm. deflection corresponded to 1/5.9 microampere. Assuming that one can detect a movement on the scale of 0.1 mm., the smallest current one could read (Δi) would be 1/59 microampere or approximately 0.02 microampere. By measurement the current flowing in the arm i_2 (Fig. 1) was found to be 5 microamperes. Substituting,

$$\text{per cent error in determining } r = \frac{2 \times 0.02}{5} \times 100 = 0.8 \text{ per cent.}$$

If one could detect a 1/20 mm. deflection this value becomes 0.4 per cent.

TABLE I

Reproducibility of results using a single suspension of yeast cells. The cells were centrifuged, washed, and suspended in 3.7 per cent dextrose solution, which was found to be isosmotic with William's solution. The ratio tabulated represents $\frac{R_2 \text{ for suspension}}{R_2 \text{ for suspending medium}}$. R_2 is measured in ohms as described in the text.

Time	Ratio	Deviation from mean	Average deviation of ratio from mean
a.m.			
10:00	1.827	+0.014	
10:10	1.815	+0.002	
10:20	1.815	+0.002	
10:25	1.805	−0.008	
10:30	1.808	−0.005	
10:35	1.806	−0.007	
10:40	1.814	+0.001	
10:45	1.807	−0.006	
10:50	1.811	−0.002	
10:52	1.807	−0.006	
10:55	1.809	−0.004	
11:00	1.818	+0.005	
11:05	1.814	+0.001	
11:10	1.814	+0.001	
11:17	1.811	−0.002	
11:20	1.819	+0.006	
11:28	1.821	+0.008	
11:35	1.812	−0.001	
11:34	1.816	+0.003	
11:44	1.817	+0.004	
Mean..........	1.813		±0.004 = 0.24 per cent

Using the relation found in the text for the per cent error in reading the ratio r, we find that if one could detect a 1/20th mm. deflection on the galvanometer this error would be 0.4 per cent. Evidently with the care exercised we could detect a slightly smaller deflection. The maximum deviation was 0.8 per cent which agrees exactly with the error in a single setting if one detected a deflection of 0.1 mm.

Attention is called to the fact that complete independence of the absolute magnitude of the resistances, R_1 and R_2, is established by the above derivation of the expression showing the per cent error in determining *r*. It is not necessary to work on the "most sensitive part" of the bridge, and the experimenter is thus able to use almost any available apparatus.

The error in determining *r* in a series of twenty readings is shown in Table I. It will be seen that both by calculation and by measurement the expected accuracy is well under 1 per cent, if the precautions outlined have been followed.

SUMMARY

A device for quickly and accurately measuring the population density of a suspension of microorganisms, permitting the preparation of yeast suspensions of known density to within 1 per cent error, was constructed with two Visitron photoelectric cells, a single light of high intensity and a good Wheatstone bridge for balancing the currents from the two photoelectric cells. A large Pyrex milk culture tube holding the suspension is placed in the path of one beam of light coming through a small longitudinal slit and thence to one photocell; a second similar slit directs another beam of light upon the second photocell, thus causing dissimilar currents to flow, the ratio of whose magnitudes may be measured by the bridge resistances. A relation between these currents and the relative light intensities is shown, and the one significant unmeasurable variable (the characteristic constant of a photocell) is practically eliminated by the use of a method of ratios.

After careful standardization of technique the apparatus proved more accurate than other methods available for the purpose indicated. In rapid use its accuracy may be put safely at 1 per cent for measuring the densities of cultures of approximately the same age and composed of cells having comparable optical characteristics.

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THE EQUILIBRIUM BETWEEN ACTIVE NATIVE TRYPSIN AND INACTIVE DENATURED TRYPSIN

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Hemoglobin denatured by acid or coagulated by heat and dissolved in acid becomes native again when brought to nearly neutral solution. From this fact the conclusion was drawn that there is an equilibrium between native and denatured protein which depends on the temperature and the pH (Anson and Mirsky, 1925). If there is such an equilibrium then at a suitable pH denaturation brought about by heating should be reversed by cooling. Reversal of heat denaturation by mere cooling was actually found to take place with serum albumin, globin, and pepsin (unpublished experiments). In none of these three cases was reversal complete, so none of these three proteins was suitable for the exact study of definite equilibrium states. A suitable protein has now been found. Trypsin, which catalyzes the hydrolysis of proteins, is itself a protein (Northrop and Kunitz, 1932). And the denaturation of trypsin is readily and completely reversible. If trypsin is heated to 60°C. in 0.05 N acid it is converted into a protein which is completely precipitable by quarter saturated ammonium sulfate. When the heated trypsin is cooled it changes back into the original form which is not precipitated by quarter saturated ammonium sulfate. If trypsin is heated or cooled to a temperature around 40°C. a definite fraction is precipitable by salt (Northrop, 1932).

Since native trypsin digests other proteins and denatured trypsin does not, the denaturation of trypsin can be followed by activity measurements. If the digestion mixture is alkaline enough and contains enough urea then there is no change of inactive denatured trypsin into active native trypsin during the measurement of tryptic activity. The same results are obtained whether denaturation is followed by measurements of activity with the urea technique or by measurements of the formation of salt-precipitable protein. In the present investigation the urea technique alone has been used both because of its simplicity and because it can be applied even to dilute solutions of trypsin. For the definition of trypsin activity units ($[\text{T. U.}]^{\text{Hb}}$) and the details of the procedure a previous paper (Anson and Mirsky, 1933) should be consulted. Hemoglobin is used as the protein substrate which is digested.

TABLE I

Effect of Acid, Alkali, and Alcohol on the Equilibrium between Native and Denatured Trypsin

Composition of solvent	Temperature of inactivation	$[\text{T. U.}]^{\text{Hb}}$ per ml. before heating × 10^4	$[\text{T. U.}]^{\text{Hb}}$ after heating and cooling × 10^4	$[\text{T. U.}]^{\text{Hb}}$ when heated to temperature of inactivation × 10^4	$[\text{T. U.}]^{\text{Hb}}$ when cooled to temperature of inactivation × 10^4	Percentage inactivation
	°C.					
0.01 N HCl	44.1	21.6	21.4	11.4	11.0	48
0.01 N HCl	44.0	199.2		99.6		50
0.003 N HCl	54.5	28.8	27.6	14.7	13.2	51.5
0.001 N HCl	61.3	28.8	28.4	14.4	15.2	48.4
0.01 N HCl in 10 per cent alcohol	38.5	28.8	28.4	14.4	14.7	49.4
0.05 N NaOH	0	345.0	260.0	17.4		95.0

Existence of Equilibria.—Under any definite conditions under which there is no irreversible inactivation, a definite fraction of the trypsin is in the active, native form and a definite fraction in the inactive, denatured form. The percentage inactivation at a given temperature is the same whether the trypsin solution is heated or cooled to that temperature (*cf.* Table I). In general the percentage denaturation at equilibrium depends only on the conditions at equilibrium and is the same whether one starts with native or denatured trypsin.

Effect of Concentration.—The concentration of a trypsin solution can be varied ten times without any effect on the percentage denaturation under given conditions (*cf.* Table I). The kinetics of denaturation are therefore the same as the kinetics of the reversal of denaturation.

Effect of pH.—In a rough way the more trypsin is ionized by either acid or alkali the more the equilibrium between native and denatured trypsin is shifted toward the denatured form. A theory of the mechanism of denaturation by acid and alkali will be presented in a paper on the equilibrium between native and denatured hemoglobin. According to this theory the effect of pH on the equilibrium between the native and denatured forms of a protein must be correlated with differences in the titration curves of the native and denatured forms. The data at present available do not permit any detailed comparison of the effects of pH on the ionization and denaturation of trypsin.

The marked effect of pH on the equilibrium between native and denatured trypsin is not apparent at room temperature. At 25°C. native trypsin is the equilibrium form even in 0.01 N hydrochloric acid. That is, denatured trypsin, if brought to 25°C. in 0.01 N hydrochloric acid, changes completely into native trypsin. The importance of pH is clear at higher temperatures. If trypsin is heated at any pH a temperature is reached at which the enzyme is half denatured. This temperature of half denaturation is very sensitive to the pH and is lowered by either acid or alkali (*cf.* Table I). Only in acid solution can the experiments be carried out without irreversible inactivation. In acid solution, as is shown in Table I, the activity after heating and cooling is the same as before heating. In alkaline solutions in which the temperature of half denaturation is low there is irreversible inactivation. In neutral solutions in which the temperature of half denaturation is high, the denatured trypsin is digested so rapidly by the native trypsin that measurements of equilibria are impossible. The irreversible inactivation by alkali and the digestion in neutral solution have been studied in detail by Northrop and Kunitz (1934).

The Effect of Temperature.—In 0.01 N hydrochloric acid trypsin is almost completely native at 40°C. and almost completely denatured at a temperature 10° higher. The logarithm of the equilibrium constant (the ratio of native trypsin to denatured trypsin) is proportional to the reciprocal of the absolute temperature as is stated by van't Hoff's relation between the heat of reaction and the effect of temperature on the equilibrium

$$\ln K = -\frac{\Delta H}{KT} + C$$

Table II gives the observed values of the percentage denaturation and the values calculated from van't Hoff's equation, assuming the heat of reaction to be −67,600 calories per mole.

The Effect of Alcohol.—In general denaturing agents shift the equilibrium between native and denatured trypsin towards the denatured form. 10 per cent alcohol in 0.01 N hydrochloric acid lowers the temperature of half inactivation 5.5° (*cf.* Table I).

We are now in a position to understand why trypsin is such favorable material for the study of denaturation and its reversal. The first condition for the reversal of denaturation is that the denaturation procedure used should not cause secondary irreversible changes. Trypsin is remarkably stable in acid. The second condition for reversal is that the denatured protein be brought to a pH at which the native form is the equilibrium form. In the case of some proteins this means a pH close to the isoelectric point, *i.e.* a pH at which the denatured protein is insoluble, at which it is precipitated before reversal of denaturation can take place. Denatured trypsin changes into native trypsin in acid solutions in which denatured trypsin is entirely soluble.

TABLE II

Effect of Temperature on the Equilibrium between Native and Denatured Trypsin in 0.01 N *Hydrochloric Acid*

Temperature	Percentage denaturation	Percentage denaturation calculated from $\ln K = -\frac{\Delta H}{KT} + C$ $-\Delta H = 67{,}600$ calories/mole
°C.		
42	32.8	32.8
43	39.2	41.0
44	50.0	50.0
45	57.4	56.4
48	80.4	80.0
50	87.8	87.2

EXPERIMENTAL

The trypsin used was prepared according to Northrop and Kunitz (1932). For the experiments of Table I the trypsin cake was simply diluted with the solvents described. For the experiments of Table II the trypsin was first dialyzed in the cold. Dialysis does not affect the results because so much solvent is added to the trypsin cake that the salt introduced with the cake is eventually present in extremely dilute solution.

The method of estimating active native trypsin in the presence of inactive denatured trypsin by the use of a suitable hemoglobin solution has already been described (Anson and Mirsky, 1933).

The experiments with dilute trypsin in 0.01, 0.003, and 0.001 N hydrochloric acid, which include the experiments on the effect of temperature on the percentage denaturation, were carried out as follows: To estimate the activity before heating 5 ml. of hemoglobin solution plus 0.5 ml. water were poured into 0.5 ml. enzyme solution and the digestion was carried out for 5 minutes. Exactly the same procedure was used to measure the activity after heating and cooling. The heating consisted in keeping the test-tube containing the enzyme solution in a water bath 2° above the inactivation temperature for 2 minutes. The cooling consisted in keeping the trypsin solution at 25° for 10 minutes. The enzyme solution was heated to the inactivation temperature by being kept in a water bath at the inactivation temperature for 2–3 minutes and cooled to the inactivation temperature by being kept first in a bath 1–2° above the inactivation temperature for 1–2 minutes and then in a bath at the inactivation temperature for 2–3 minutes. The time at the higher temperature sufficed to produce more than half denaturation as was determined by separate experiments.

In the case of the ten times more concentrated trypsin in 0.01 N hydrochloric acid, the enzyme was first diluted ten times and a digestion mixture of the normal composition obtained by adding to 0.5 ml. of trypsin solution 4.5 ml. of a mixture of 10 parts hemoglobin solution and 0.8 parts water and then there were added to 1 ml. of the resulting solution 5 ml. of a mixture of 5 parts hemoglobin solution and 1 part water. The digestion time was measured from the first addition of hemoglobin.

In the case of the solution of trypsin in acid alcohol, the enzyme solution was kept at the inactivation temperature for 5 minutes and before being cooled to the inactivation temperature it was kept at a temperature 1.5° higher for 1 minute.

To estimate the activity in 0.05 N sodium hydroxide at 0°C. 1 ml. of 0.1 N sodium hydroxide was added to 1 ml. trypsin solution. After 1 minute there were added to 0.5 ml. of this solution a mixture of 5 ml. hemoglobin solution and 0.5 ml. 0.05 N hydrochloric acid. Digestion was carried out for 5 minutes. To find out how much of the trypsin was reversibly inactivated by the alkali, 14.5 ml. of 0.01 hydrochloric acid were added to 0.5 ml. of the alkaline trypsin and, after 10 minutes at 25°C., 5 ml. of hemoglobin solution were added to 1 ml. of the acidified trypsin and digestion was carried out for 5 minutes.

SUMMARY

There is a mobile equilibrium between the native and denatured forms of trypsin which depends on the concentrations of acid, alkali, and alcohol and on the temperature.

The heat of denaturation in 0.01 N hydrochloric acid calculated from the effect of temperature on the equilibrium constant is −67,600 calories per mole.

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THE EQUILIBRIA BETWEEN NATIVE AND DENATURED HEMOGLOBIN IN SALICYLATE SOLUTIONS AND THE THEORETICAL CONSEQUENCES OF THE EQUILIBRIUM BETWEEN NATIVE AND DENATURED PROTEIN

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The denaturation of hemoglobin by acid is partially reversible (Anson and Mirsky, 1931). If acidified hemoglobin is rapidly neutralized all the protein is precipitated. If the acidified hemoglobin is first made slightly alkaline and then after a few seconds brought to the neutral point only a third of the protein is precipitated. The soluble two-thirds has again the properties of native hemoglobin.

Equilibria in Salicylate Solutions.—It has already been shown (Anson and Mirsky, 1929 *b*) that concentrated sodium salicylate in neutral solution denatures hemoglobin and keeps denatured hemoglobin in solution. It will be shown in this paper that denaturation by salicylate is completely reversed when the salicylate is removed by dialysis under suitable conditions or when the salicylate solution is simply diluted with water. Salicylate not concentrated enough to denature hemoglobin completely produces an equilibrium mixture of native and denatured hemoglobin. The higher the salicylate concentration the higher is the percentage denaturation (see Fig. 1). At any given salicylate concentration the percentage denaturation is the same whether one starts with native or with denatured hemoglobin. Decreasing the hemoglobin concentration from 1 per cent to 0.5 per cent or raising the temperature from 25°C. to 35°C. has no detectable effect on the equilibrium in 0.25 M salicylate solution.

Differences between Native and Denatured Hemoglobin.—Hemoglobin denatured by salicylate has three properties characteristic of hemoglobin denatured by other means. It is insoluble under the same conditions under which native hemoglobin is soluble; it is digested by trypsin which does not attack native hemoglobin; and it has the parahematin type of spectrum which is also given by a solution of hemin in pyridine. When the denaturation of hemoglobin by salicylate is reversed, the original properties of native hemoglobin are restored.

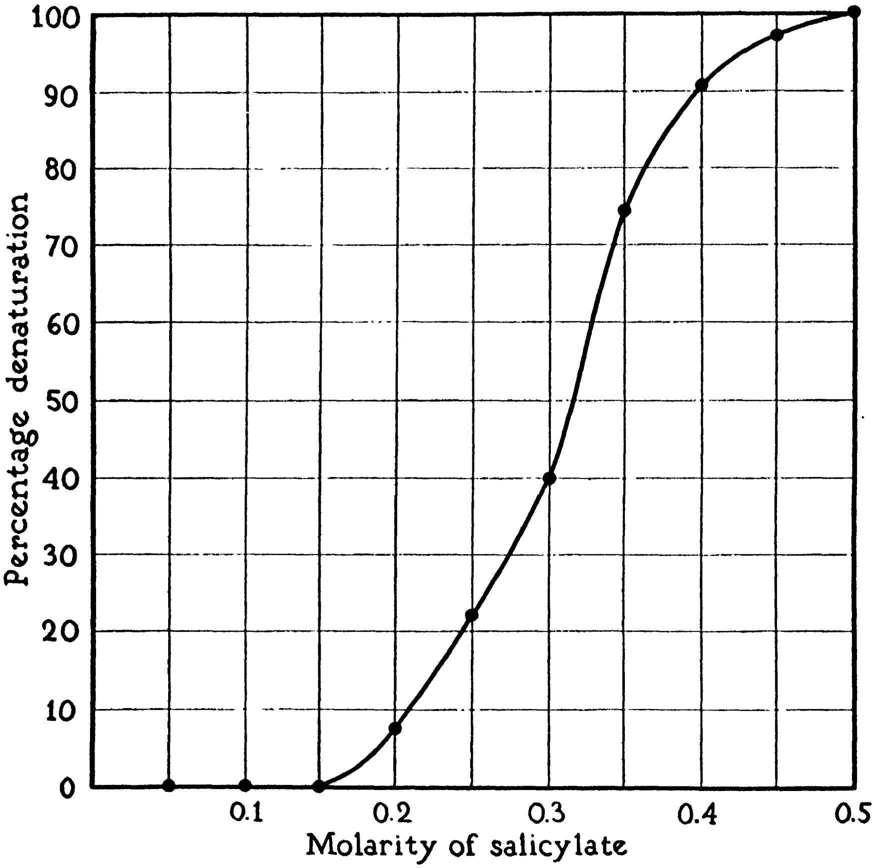

FIG. 1. The effect of salicylate concentration on the denaturation of hemoglobin

One can, if one so wishes, assume that salicylate converts hemoglobin not into denatured hemoglobin but into some other compound which also is insoluble, and digestible and has the parahematin spectrum. Before such an assumption need be considered seriously some difference between the hypothetical other compound and denatured hemoglobin must first be demonstrated.

The Change in Spectrum on Denaturation.—The change in spectrum resulting from the denaturation of hemoglobin is the basis of the method used to estimate the percentage denaturation. Hemoglobin consists of a native protein, globin, joined to an iron porphyrin complex, heme. The spectrum of heme is changed by combination of heme with native globin and the spectrum of the heme-globin compound is changed when the globin is denatured. The denaturation of hemoglobin can, therefore, be followed spectroscopically, heme acting as an indicator of the change in the protein with which it is combined. This was first pointed out for the compounds of reduced (ferrous) heme. The compound of reduced heme and native globin has the spectrum of reduced hemoglobin while the compound of reduced heme with denatured globin has the spectrum of hemochromogen (Anson and Mirsky, 1925; 1928). Analogously the compound of oxidized heme and native globin has the spectrum of methemoglobin while the compound of oxidized heme and denatured globin has the spectrum of parahematin (Keilin, 1926). Parahematin has no distinct absorption in either the yellow or the red, whereas alkaline methemoglobin and hematin have a band in the yellow and acid methemoglobin and hematin have a band in the red. The spectrum of parahematin is thus qualitatively different from the spectra of the other hemoglobin derivatives.

Because of technical difficulties the spectroscopic study of the denaturation of hemoglobin and its reversal has not been satisfactory. The difficulties are these. In neutral solution both globin hemochromogen and globin parahematin are insoluble. In alkaline solution, globin hemochromogen prepared from hemoglobin can combine with extra reduced heme and globin parahematin dissociates to a greater or lesser extent into denatured globin and oxidized heme. It is doubtful whether the pure spectrum of globin parahematin has hitherto been observed. In the neutral salicylate solutions used in the present experiments globin parahematin is neither precipitated nor dissociated and so the difficulties which have been mentioned are avoided.

The Change in Absorption of Green Light on Denaturation.—Viewed in the monochromatic green light easily obtained by the use of color filters from the mercury arc lamp, all pigments appear to be of the same color. Different pigments, however, differ in the extents to which they absorb the green light. Denatured methemoglobin prepared by the addition of salicylate to a neutral phosphate solution of native methemoglobin absorbs the green light about twice as strongly as does native methemoglobin. Hence one can measure the extent of denaturation of methemoglobin by measuring the extent of absorption of green light with a colorimeter. The greater the percentage denaturation, the greater the absorption of green light.

Aggregation.—The solubility of denatured hemoglobin in dilute neutral salicylate solution is limited. If the protein concentration is too high there results, first, association or aggregation of the protein molecules, and then visible precipitation. Aggregation of the molecules of denatured hemoglobin increases the absorption of green light by an equilibrium mixture of native and denatured hemoglobin in two different ways. First, the aggregated pigment has a greater absorbing power than the unaggregated. Secondly, if as a result of aggregation denatured hemoglobin is removed from the equilibrium mixture

$$\text{unaggregated native hemoglobin} \leftrightarrows \text{unaggregated denatured hemoglobin}$$

then more denatured hemoglobin is formed to maintain the equilibrium and there is an increase in the total amount of denatured protein and hence in the light absorption. This complicating effect of aggregation on the study of an equilibrium has already been discussed in connection with the equilibrium

$$\text{reduced heme} + \text{pyridine} \leftrightarrows \text{pyridine hemochromogen}$$

(Anson and Mirsky, 1929*a*; 1930). The same formation of denatured from native hemoglobin which takes place when the denatured hemoglobin is aggregated also takes place when the denatured hemoglobin is digested.

To avoid aggregation in experiments on the effect of salicylate on the equilibrium between native and denatured hemoglobin, the hemoglobin concentration is kept as low as is consistent with accurate colorimetric measurements. Bovine hemoglobin is used because compared with hemoglobin from other common animals it is relatively soluble and requires a relatively high concentration of salicylate for denaturation. The solutions cannot be made more alkaline to avoid aggregation because the optical properties of the pigments change and become much more alike than they are in neutral solution.

If salicylate is added to native methemoglobin the absorption of green light at first increases rapidly with time, then remains constant, and finally increases again very slowly. If the same final conditions are obtained by the addition of water to denatured methemoglobin in concentrated salicylate solution then the light absorption first decreases rapidly, then remains constant, and finally increases very slowly. The value of the light absorption which is constant for a while is the same whether the experiment is started with native or with denatured hemoglobin. These results, which are given in Table I, suggest that a true equilibrium is measured before slow aggregation or other change takes place. Aggregation, however, is not definitely

TABLE I

Effect of Salicylate Concentration and Time on the Absorption of Green Light by Hemoglobin Solutions

Molarity of salicylate	Time, *min.*							
	0.5	5	10	15	30	40	60	120
0	20				20			
0.05 (*a*)				20.2		20.1		
0.10 (*a*)				20.1		20.1		
0.15 (*a*)		20.0		20.0				
(*b*)		20.0		20.0				
0.20 (*a*)	20.0	19.7	19.2	19.1	18.7	18.7	18.0	17.5
(*b*)	18.0	18.7	18.7	18.7	18.7	18.3	17.4	17.4
0.25 (*a*)	18.0	17.3	16.9	16.8	16.7	16.7	16.0	16.0
(*b*)	15.7	16.7	16.7	16.7	16.7	16.0	16.0	16.0
0.30 (*a*)	17.0	15.1	14.7	14.7	14.6	14.5	14.0	13.8
(*b*)	12.3	14.2	14.7	14.7		14.6	13.9	13.9
0.35 (*a*)	13.5	12.3	11.8	12.0	12.0	12.0	11.8	11.8
(*b*)	11.3	12.0	12.0	12.0	11.9	12.0	11.8	11.8
0.40 (*a*)	13.0	11.2	11.0	11.0				
(*b*)	10.8	11.0		11.0				
0.45 (*a*)	13.0	11.0	10.7	10.7				
0.50 (*a*)		10.5			10.5			

The figures represent colorimeter readings. (*a*) means that the equilibrium was reached by the formation of denatured hemoglobin, (*b*) by the formation of native hemoglobin. (See experimental part of text.)

excluded at any stage of the reaction. The state of dispersion of the protein can be decided conclusively only by direct molecular weight determinations.

That there is an equilibrium between a red form and a brown form of hemoglobin is an observed fact whose validity does not depend on the existence or non-existence of aggregation. If aggregation does take place before the equilibrium can be reached and measured then one cannot tell from the total effects of salicylate concentration and temperature on the percentage denaturation to what extent the salicylate concentration and the temperature influence the degree of aggregation and to what extent they influence the equilibrium between unaggregated native and denatured hemoglobins. If, on the other hand, the equilibrium is not being disturbed by aggregation then any theory of denaturation must be in harmony with the facts first, that the curve relating percentage denaturation to salicylate concentration is S-shaped (see Fig. 1) and, secondly, that temperature has little effect on the equilibrium between native and denatured hemoglobin although it has a great effect on the equilibrium between native and denatured trypsin (Anson and Mirsky, 1934). The theory of denaturation we shall now present which is in harmony with the facts which have just been stated is simply a restatement in other words of the existence of an equilibrium between the native form of a protein N and its denatured form, D.

Let us suppose that there is added to the equilibrium mixture $N \rightleftarrows D$, a substance, X, which can combine reversibly with both N and D or modify N and D in any reversible way. There then results the double equilibrium

$$\begin{tikzcd}
N + X \arrow[r, leftarrow, shift left] \arrow[r, shift right] \arrow[d, shift right] & NX \arrow[d, shift left] \\
D + X \arrow[u, shift right] \arrow[r, leftarrow, shift left] \arrow[r, shift right] & DX \arrow[u, shift left]
\end{tikzcd}$$

The total native protein is $N + NX$, the total denatured protein $D + DX$. There are two ways in which the equilibria can be influenced reversibly. First, the equilibrium constants can be changed by a change in the solvent, in the temperature, or in the rate at which any form of energy is being absorbed. Secondly, the amounts of N and D combined with X can be increased by an increase in the concentration of X. If X has the same affinity for D as for N, then the fraction of D converted into DX by the addition of X is the same as the fraction of N converted into NX and there is no change in the total percentage denaturation. If X has a greater affinity for D than for N, relatively more D is converted into DX than N into NX and the percentage denaturation is increased. The necessary relation between the denaturation equilibria and the X combination equilibria is given by the identity:

$$\frac{N/D}{NX/DX} \equiv \frac{N/NX}{D/DX}$$

Given the mere fact that the addition of X causes denaturation one cannot decide whether the cause of the denaturation is a change in the equilibrium constants of the individual equilibria or a greater combination of X with D than with N, or whether X acts in both ways. The decision must be made on the basis of measurements of the individual equilibria or on the basis of chemical probabilities. It is likely, for instance, that a change in percentage denaturation brought about by acid is due to a difference in the affinities of acid for the native and denatured forms of the protein.

In practice when X is added to a protein there usually results a whole series of X compounds and hence the complicated equilibrium.

$$\begin{tikzcd}
N \arrow[r, shift right] \arrow[d, shift right] & NX_1 \arrow[l, shift right] \arrow[r, shift right] \arrow[d, shift right] & NX_2 \arrow[l, shift right] \arrow[d, shift right] & \cdots\cdots & NX_n \arrow[d, shift right] \\
D \arrow[r, shift right] \arrow[u, shift right] & DX_1 \arrow[l, shift right] \arrow[r, shift right] \arrow[u, shift right] & DX_2 \arrow[l, shift right] \arrow[u, shift right] & \cdots\cdots & DX_n \arrow[u, shift right]
\end{tikzcd}$$

The shape of the curve relating the concentration of X to the percentage denaturation depends on the values of all the equilibrium constants. By substituting a suitable set of values into the involved equation representing the complicated equilibrium one can obtain the S-shaped curve which relates the concentration of salicylate to the percentage denaturation of hemoglobin by salicylate. Such curve fitting is of little theoretical significance so long as the numerous equilibrium constants are chosen arbitrarily. What are needed are X combination curves to go with X denaturation curves; for instance, acid titration curves to go with measurements of the effect of pH on denaturation. The presentation of the detailed mathematical formulation of the theory of equilibria had best be postponed until data for testing the equations are available.

The heat of the denaturation $N \rightarrow DX$ caused by the addition of X to N is equal to the heat of the reaction $N \rightarrow NX$ plus the heat of the reaction $NX \rightarrow DX$. Since the reaction $N \rightarrow NX$ may be either endothermic or exothermic depending on the nature of X, the heat of the denaturation $N \rightarrow DX$ will vary with the nature of the denaturing agent.

EXPERIMENTAL

Equilibria.—The stock solutions are a freshly filtered 1 per cent solution of dialyzed bovine methemoglobin prepared according to Anson and Mirsky (1931) in a buffer made up of equal parts 0.1 M K_2PO_4 and 0.1 M KH_2PO_4 and a filtered 1 M solution of sodium salicylate which is stored in the cold. Only such a sample of salicylate should be used which on filtration yields a water-clear solution. Monochromatic green light for the colorimetric measurements is obtained from the mercury vapor lamp by means of the two color filters supplied by the Corning Glass Company for the isolation of the green line. The standard which is set at 20 is made up by adding 9 parts of water to 1 part of the hemoglobin solution. All the experiments described in the experimental part are carried out at 25°C.

To reach the equilibrium corresponding to X tenth molar salicylate solution by formation of denatured hemoglobin, 10 ml. of solution are made by adding X ml. of the 1 M salicylate to a mixture of 1 ml. hemoglobin and $10 - (X + 1)$ ml. water. To reach equilibrium from the other side by the formation of native hemoglobin, 10 ml. of solution are made by adding X ml. of salicylate to a mixture of 1 ml. hemoglobin and $7 - (X + 1)$ ml. water and then after 3 minutes adding 3 ml. more of water. The colorimetric readings at various times are given in Table I.

Since denatured methemoglobin absorbs green light $\frac{20}{10.5}$ or 1.9 times as strongly as native methemoglobin, the relation between the colorimetric reading, R, and the fraction, D, of the protein which is denatured is given by

$$(1 - D) + 1.9D = \frac{20}{R}$$

or

$$D = \frac{20 - R}{0.9R}$$

The greater the percentage denaturation, the less accurate is the estimation of the percentage denaturation by this colorimetric method. Fig. 1 shows the relation between the salicylate concentration and the percentage denaturation.

The Digestion Test.—Hemoglobin in salicylate solution is not digested by trypsin unless the salicylate concentration is high enough to cause some denaturation as shown by the optical test. If a 1 per cent solution of hemoglobin in 0.3 M salicylate, which by the colorimetric test is about 40 per cent denatured and 60 per cent native, is diluted with equal volume of water and placed in boiling water the protein precipitates. If 1.7 × 10^{-3} hemoglobin units (Anson and Mirsky, 1933) of trypsin are added to each ml. of the solution before it is diluted and heated, then digestion of the denatured hemoglobin takes place, more denatured hemoglobin is formed to maintain the equilibrium and so on until after 20 minutes no precipitate is formed if the solution is diluted and heated. A 1 per cent solution of hemoglobin in 0.1 M salicylate which by the colorimetric test is all native even after 20 minutes treatment with trypsin yields the same sort of precipitate on dilution and heating as does hemoglobin not treated with trypsin. The dilution is made with 0.2 M salicylate instead of with water so that the final salicylate concentrations of the solutions which are heated are 0.15 M in the two cases. Native hemoglobin prepared from denatured hemoglobin behaves like native hemoglobin which was never denatured. To denature the hemoglobin 1 ml. of salicylate is added to 1 ml. of hemoglobin. Brown native hemoglobin is obtained again from the red denatured hemoglobin by the gradual addition of 7.5 ml. water. Finally 0.5 ml. trypsin solution is added. Since trypsin is probably destroyed under the conditions under which hemoglobin is inactivated there is probably more active trypsin in the solution of native hemoglobin which is not digested than in the solution of denatured hemoglobin which is digested.

The Solubility Test.—Denatured methemoglobin is insoluble in 0.3 saturated ammonium sulfate. 1 ml. salicylate is added to 1 ml. hemoglobin and the protein is then precipitated by the addition of a mixture of 12 ml. water and 6 ml. saturated ammonium sulfate. Native hemoglobin is soluble under the same final conditions. No precipitate results from the addition of 6 ml. saturated ammonium sulfate to a solution prepared by adding 1 ml. hemoglobin to a mixture of 12 ml. water and 1 ml. salicylate. "Reversed" hemoglobin behaves like native hemoglobin. 1 ml. salicylate is added to 1 ml. hemoglobin to bring about denaturation; 12 ml. are then added gradually to bring about the reversal of denaturation. When finally 6 ml. of saturated ammonium sulfate are added only a slight haze is obtained. The clear brown filtrate from this solution stays clear.

SUMMARY

The denaturation of hemoglobin by salicylate in neutral solution is completely reversible.

There is a mobile equilibrium between native and denatured hemoglobin in neutral salicylate solution. The higher the salicylate concentration the greater is the percentage denaturation.

When there is a mobile equilibrium between the native and denatured forms of a protein, denaturation is caused by the addition of any substance which has a greater affinity for the denatured than for the native form.

Theoretically the heat of denaturation must vary with the denaturing agent and must depend on the heat of combination of the denaturing agent with the protein.

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ON THE RELATION BETWEEN TOXICITY, RESISTANCE, AND TIME OF SURVIVAL, AND ON RELATED PHENOMENA

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The problem of the relation between toxicity, resistance of organisms to noxious agents, and time at which the expected event occurs, can be considered from two aspects. Under the usual experimental conditions the action of a destructive agent is observed on a group of organisms and therefore, as with any collective phenomenon, the order of events (that is, in the present case the shape of the mortality curve) is the most conspicuous attribute of the phenomenon. Several authors, interested in this problem, have believed that the number of organisms present in the group actually determined the rate at which the organisms died (1–10). Another group (11–17) of investigators focused interest on the fundamental phenomenon, considering the relation between toxicity, resistance, and time of survival as though only a single organism were involved. They attribute the particular shape of the mortality curve to a particular distribution of the resistance among the individuals. Both aspects have been discussed extensively in the literature. Many theories and equations have been proposed none of which seems to agree with all the different types of experiments. It appears superfluous to review the literature, for several extensive reviews of the subject have been published lately (18–21). All theories which assume that the number of organisms present in the system influences the fundamental process must be rejected on the basis that the action of an agent on one organism (*e.g.*, higher animal) must be independent from that on another, provided that the agent is present in sufficient quantities not to be used up to an appreciable extent in the process. Those who believed that the peculiar death order of organisms is due to individual differences in the resistance of the organisms, had to assume a peculiar distribution of the resistance and even then it could not be explained why sometimes a more or less symmetrical distribution of the survival time of the organisms changes to an asymmetrical one, when the strength of the destructive agent is changed.

An explanation for this was sought in the fact that the fundamental relation between toxicity, resistance, and time is not necessarily a linear one. Thus, even if the resistance showed a normal (symmetrical) variation, the distribution of the survival time might be asymmetrical. A general equation, describing the fundamental relationship, has been derived and set forth in a preliminary paper (22). It is based on the experimental fact that toxicity has a threshold value which can be considered as corresponding to a state of equilibrium (stationary state) between toxicity and resistance and on the assumption that the variation of the time is proportional to the relative variation of the difference between toxicity and resistance. Among the equations which have been proposed to account for the relation between toxicity, resistance, and time of survival (Deyer and Walker (23) (*cf.* the criticism offered by Glenny (24)), Powers (25), Carpenter (26), Ponder (27)) there are some which are non-linear. That of Ponder (27) resembles most nearly the equation given in the preliminary paper. However, differences in the two equations seem to account sufficiently for differences in the applicability of the two equations. In more recent papers Ponder (28) abandoned his first equation for a new one which is claimed to have more general applicability.

Special forms of the equation given in a general form in the preliminary paper are developed below. There are, altogether, four independent variables; resistance (*r*), toxicity (*h*), time (*t*), and temperature (*T*), and experiments can be so conducted that two of them vary and two remain constant. In addition, to each value of resistance, there corresponds a number of organisms (the percentage killed) expressing the probability that any one of the organisms has a resistance equal to or lower than the one in question. The following combinations of the four variables are selected for the test of the theory:

(1) The resistance and the time vary while the temperature and toxicity are kept constant (*cf.* Section II below). (*a*) A resistance value can be calculated from the time which is required to kill a certain number of organisms. The resistance of the organisms killed in this time is lower than, or at most, equal to, this value. The percentage of organisms killed can now be plotted against resistance instead of time and the shape of the curve thus obtained can be analyzed. It will be demonstrated (Section II) that, as far as one can judge from experiments involving a large error, the mortality-resistance curves correspond to a normal frequency distribution even in cases in which the mortality-time curves are asymmetrical. (*b*) It will also be demonstrated by means of an ideal example that if one attributes to the resistance of an organism values which vary around an arbitrary average according to the probability rule, times of survival can be calculated on the basis of the theory. Percentage mortality plotted against these time values will show the same peculiar order of death of organisms as do the experimental curves. Furthermore, it is shown in ideal examples (which will be referred to in the future as "theoretical curves") that an increase of toxicity decreases the asymmetry as it does in experimental cases. (2) It is of great interest to test the theory on cases which are not complicated by statistical phenomena. Such cases are those in which the resistance is kept constant (*cf.* Section III). There are many experiments available in which the resistance and the temperature are kept constant while the toxicity and time of survival are varied. To be able to test the theory in these combinations it is necessary to assume a relation between the toxicity of an agent and its concentration. It is obvious that whatever this relation may be, it must approach a linear relation if the variation is limited to a sufficiently small range of concentration. For larger ranges of concentration the theory gives good agreement with the experiments if it is assumed that for unicellular organisms the toxicity is proportional to the adsorbed amount of the agent. (3) Cases are discussed in which the time and the temperature are kept constant and the toxicity (concentration of the toxic agent) and the resistance vary (*cf.* Section IV). In consequence the mortality changes with the concentration. (4) The relation between time of survival and temperature is studied in cases in which the toxicity and resistance are kept constant (*cf.* Section V).

A simple relation between dose and toxicity for higher animals has so far not been found. In this paper the test of the theory has been restricted to the action of various agents on unicellular organisms.

I. The Fundamental Relationships

Most of the different equations which have been proposed to express the relation between toxicity and the resistance of an organism do not account for the fact that at a certain, still finite value of toxicity, its effect becomes unnoticeable and the time of survival becomes infinite,[1] (assuming an ideal, otherwise non-noxious environment). To account for this one has to assume that the noxious power (h) and the resistance (r) are in a state comparable to an equilibrium when the survival time becomes infinite in spite of the fact that the noxious power is greater than zero. In other words, the threshold value of toxicity is determined by the equilibrium. The parameter ξ on which the time depends can be considered as the difference between noxious power and resistance and the equilibrium (or stationary state) may be characterized by—

$$\xi = h - r = 0;\ t = \infty$$

This condition is fulfilled if

$$t = f\left(\frac{1}{\xi}\right) = f\left(\frac{1}{h-r}\right)$$

The simplest assumption about the function f is that the variation of the time is proportional to the relative variation of the parameter ξ (the difference between toxicity and resistance). Thus

$$(1)\quad dt = -a\frac{d\xi}{\xi} = -a\frac{d(h-r)}{h-r}$$

and by integration

$$(2)\quad t = -a\ln\xi + K = -a\ln(h-r) + K$$

where a and K are constants. K, the integration constant, fixes the zero point of the time scale and a has the character of the reciprocal of a velocity constant. It determines the specific effect of the parameter ξ on the time in any special case. The actual numerical value of these constants naturally depends on the units in which time, disinfecting power, and resistance are measured.

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[1] The acceptance of the "phenol coefficient" as a relative measure of the disinfecting power involves the assumption that the time required to kill is inversely proportional to the disinfecting power and proportional to the resistance. This relation has also been used by Reichel (29). It is, however, untenable for, if it were true, the same time of survival should correspond to a given ratio of resistance and disinfecting power regardless of the organism or agent used. It will be shown that this is not the case.

II. The Variation of the Time with the Variation of the Resistance If the Temperature and Toxicity Are Kept Constant

(*a*) As a first application of Equation 2, the variation of the resistance should be calculated for a given variation of the time in the case of constant disinfecting power and constant temperature. Since there is no way to determine independently the resistance r which is defined by Equation 2, Equation 2 cannot be tested directly in this combination of the variables. It is, however, of interest to compare the relation between percentage mortality and resistance with that of percentage mortality and time and see whether or not the asymmetry characteristic of the mortality-time curves will persist in the mortality-resistance curves. The comparison is easily achieved graphically by plotting the percentage mortality first against the time and then against the resistance calculated from the observed time by means of Equation 2. To calculate r Equation 2 must be transformed so that K is expressed with arbitrary values of t and r However, for $t = 0$, r may be $-\infty$. Therefore, it is most convenient to select for the fixed point the median time, $t_{\text{med.}}$, and the corresponding median resistance, $r_{\text{med.}}$. The relation of any other t to any other r can be calculated if Equation 1 is integrated between $t_{\text{med.}}$ and t, that is between $r_{\text{mod.}}$ and r.

$$(3) \quad \int_{t}^{t_{\text{med.}}} dt = -a \int_{r}^{r_{\text{med.}}} \frac{d(h-r)}{h-r}; \; t_{\text{med.}} - t = a \ln \frac{h-r}{h-r_{\text{med.}}} = a' \ln (h' - r')$$

The median time can be defined in the following way: Let us imagine the organisms to be arranged according to their resistance and then divide the entire group of organisms into two groups of identical size. The median time is the time during which the last organism of the group, thus arranged, containing the lower resistances, would die, but the first organism of the group containing the larger resistances, would survive. The median resistance is the corresponding value of resistance. The median time can be determined graphically. In view of the fact that, in some of the experiments, records of determinations around the median time are very scarce, and since this would make the graphic evaluation of the median time somewhat arbitrary, it seemed preferable in such cases to use the statistical average instead of the median. This was calculated in the usual way. The total number of organisms dying in each time interval was multiplied with the mean of this time interval, these products were summed, and the sum divided by the total number of organisms in the group. Where used, the average time is also designated by $t_{\mathrm{med.}}$ in the formulas and tables, thus indicating that the correct procedure would be to use the median time if there were a sufficiently accurate method for its evaluation.

It must be emphasized that a' values obtained by using Equation 3 are different for different concentrations of the toxic agents. Since the value of a depends on the units in which h and r are measured, the values of a' will depend on $h - r_{\mathrm{med.}}$, whereas a is independent of this entity. The values of a and a' will be numerically equal if $h - r_{\mathrm{med.}}$ equals unity. The a' values can be calculated on the basis of the assumption that the distribution of r is symmetrical around $r_{\mathrm{med.}}$. Whence

$$(4)\quad h - r_o = 2(h - r_{\mathrm{med.}});\ t_{\mathrm{med.}} = a' \ln 2;\ \text{and}\ a' = \frac{t_{\mathrm{med.}}}{0.6931}$$

Values for a must be estimated from experiments in which r (*e.g.*, the maximum probable resistance of a group containing a certain number of organisms) is constant and h (*e.g.*, the concentration of a disinfecting agent) is varied. Mortality-resistance (r') curves, obtained for different concentrations of the toxic agent, would, according to the theory, all be identical if a' were used for the calculation of $h' - r'$. If, however, a is used for all concentrations the interesting effect of the concentration of the toxic agent on the shape of the mortality-time and mortality-resistance curves becomes apparent.[2]

Values which were calculated from experiments by Henderson Smith (30) on the action of 0.4, 0.5, 0.6, and 0.7 per cent of phenol on *Botrytis* spores are given in Tables I to IV and Figs. 1 to 3. The value a' was taken as $t_{\mathrm{med.}}/0.6931$ from the experiment with 0.4 per cent phenol and was used also for higher concentrations. In Section III the same value was assigned to a for the calculation of the time and calculation of the median resistance with satisfactory results. It will be seen that in accordance with what has been said about the conditions under which a' equals a, $r_{\mathrm{med.}}$ is about 0.2 in terms of concentration of phenol, that is, about half of the concentration (0.4) for which a' has been calculated.

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[2] If for example, the disinfecting power is expressed in units of the median resistance, a' and a should be identical whenever the disinfecting power is double the resistance. Thus, a can be evaluated according to Equation 3 from that mortality-time curve in which the disinfecting power is practically the double of the average resistance. The value of a has been thus calculated in Tables I to IV and used for the calculation of mortality-resistance curves.

TABLE I

Action of 0.4 Per Cent Phenol on Botrytis Spores (30)

$a = 91 \qquad t_{\text{med.}} = 92.9$ min.

t	$e^{\frac{t_{\text{med.}} - t}{a}}$ *	Spores killed
min.		*per cent*
30	1.995	0
50	1.603	7.1
70	1.288	17.2
80	1.153	34.1
90	1.032	44.7
100	0.926	61.7
110	0.827	73.7
120	0.743	75.8
130	0.665	83.3
140	0.596	92.7
150	0.533	94.6
160	0.478	96.9
170	0.428	98.2

* The variation of these values (occurring in Tables I to VI) is proportional to the variation of the resistance but has the opposite sign. They are equal to $\frac{h - r}{h - r_{\text{med.}}}$. The latter expression was used for the corresponding columns in Tables VII and VIII (*cf.* Equation 3).

TABLE II

Action of 0.5 Per Cent Phenol on Botrytis Spores (30)

$a = 91 \qquad t_{\text{med.}} = 49.91$ min.

t	$e^{\frac{t_{\text{med.}} - t}{a}}$	Spores killed
min.		*per cent*
0	1.714	0
20	1.390	5.4
40	1.113	26.9
60	0.893	71.1
80	0.718	91.6
100	0.577	97.7
120	0.463	98.1
140	0.371	99.2
160	0.298	99.3

The numerical value of a' is therefore here approximately the same as that of a. A closer approximation could be attempted only if the experimental errors were smaller. In Figs. 1 to 3, the percentage mortality is plotted against the time and against $h' - r'$, that is, the resultant toxicity, which varies like the negative value of the resistance ($-r'$) when h' is constant. Points corresponding to $t_{\text{med.}}$ and $r'_{\text{med.}}$ were made to coincide. Increasing times as well as decreasing $h' - r'$ values (analogous to increasing resistance) were plotted on the abscissa. The asymmetry of the mortality-time curve as compared with the mortality-resistance

TABLE III

Action of 0.6 Per Cent Phenol on Botrytis Spores (30)

$a = 91 \qquad t_{\text{med.}} = 13.57$ min.

t	$e^{\frac{t_{\text{med.}} - t}{a}}$	Spores killed
min.		*per cent*
0	1.162	0
3	1.125	4.7
6	1.088	6.7
9	1.051	23.4
12	1.018	33.4
15	0.983	54.7
18	0.953	83.2
21	0.923	86.2
24	0.894	97.4
27	0.863	99.2
30	0.836	99.6

TABLE IV

Action of 0.7 Per Cent Phenol on Botrytis Spores (30)

$a = 91 \qquad t_{\text{med.}} = 2.92$ min.

t	$e^{\frac{t_{\text{med.}} - t}{a}}$	Spores killed
min.		*per cent*
0	1.033	0
1.5	1.017	28.7
2.5	1.005	47.9
3.5	0.994	64.0
4.8	0.980	80.8
8.0	0.946	97.9
11.25	0.913	98.4

curve is demonstrated by the fact that to the left of the median line as well as to the right of the median line the mortality-time curve is always below the mortality-resistance curve which is practically symmetrical. Figs. 2 and 3 show that the difference between the two types of curves becomes more and more negligible if h' increases, due to the fact that the variation of the resistance becomes negligible as compared to the difference between toxic power and resistance.

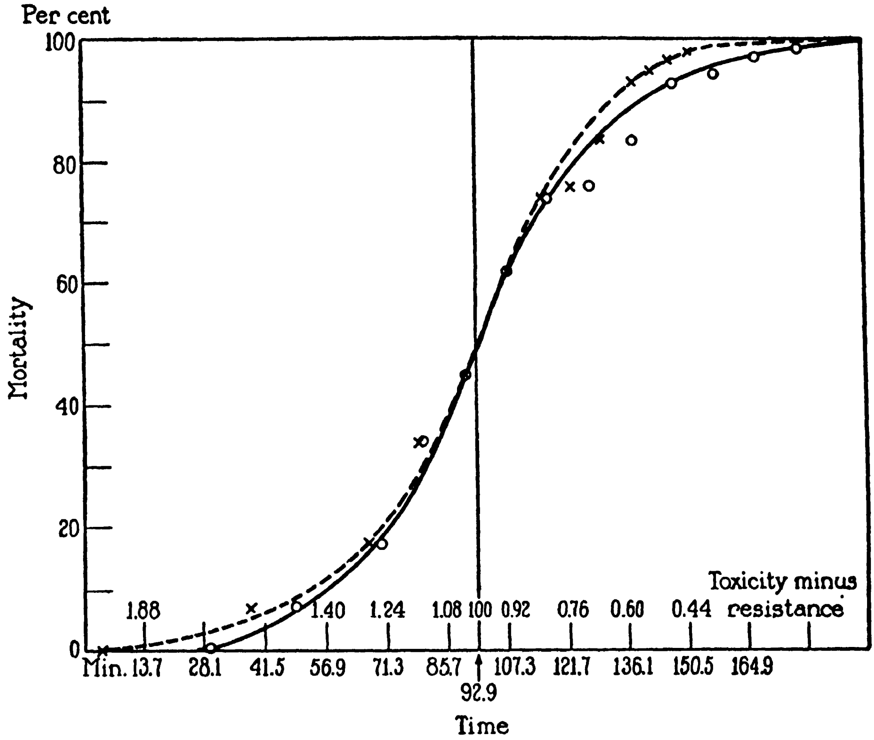

FIG. 1

FIGS. 1, 2, and 3. Curves showing that asymmetrical mortality-time curves (———), obtained from experiments on *Botrytis* spores (30) with different concentrations of phenol (0.4, 0.5, and 0.7 per cent) become symmetrical (- - - - -) if the mortality is plotted against $e^{\frac{t_{\text{med.}} - t}{a}}$.

The curves become steeper in agreement with the fact that the relative deviation is smaller, that is, the precision is greater.

There is a slight asymmetry noticeable in the mortality-resistance curves. This is due to systematic errors for which no correction has been made. It was noted by Smith, and is probably true for most experiments of this type, that a certain number of spores die during the time of an experiment, even though they are not in contact with phenol. This spontaneous death of the spores amounts

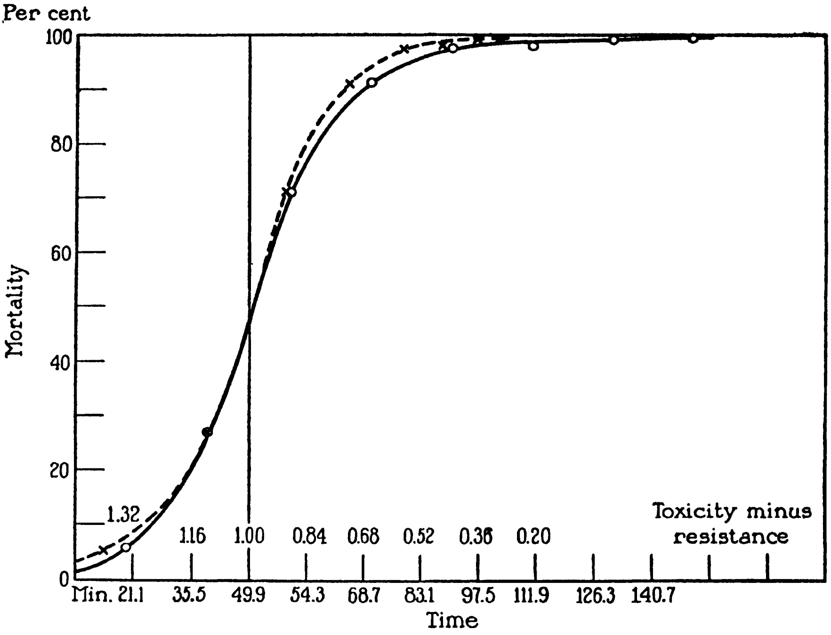
Per cent
100
80
60
40
20
0
Mortality
1.32
1.16
1.00
0.84
0.68
0.52
0.36
0.20
Toxicity minus resistance
Min. 21.1
35.5
49.9
54.3
68.7
83.1
97.5
111.9
126.3
140.7
Time

FIG. 2

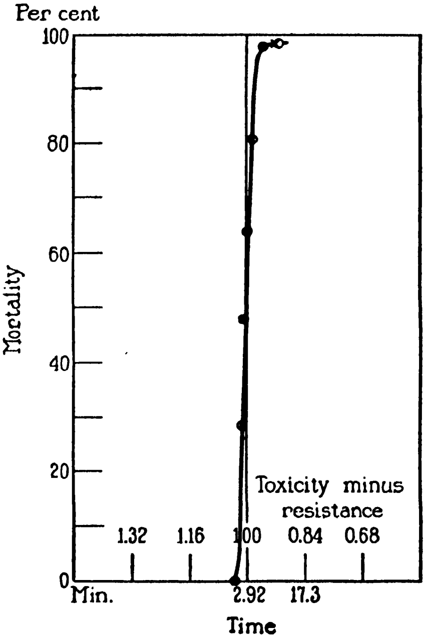
Per cent
100
80
60
40
20
0
Mortality
Toxicity minus resistance
1.32
1.16
1.00
0.84
0.68
Min.
2.92
17.3
Time

FIG. 3

to 10 to 20 per cent in some cases. In time intervals in which the death rate due to the action of the disinfectant is small (as at the beginning of an experiment) the spontaneous death rate may cause a great error.[3]

In Table V, values are given which were calculated from Chick's (4 *b*) experiments on phenol and *staphylococci*. The corresponding curves in Fig. 4 show the difference in the symmetry of the two types of curves very distinctly.

Still greater is the difference between mortality-time and mortality-resistance curves in the case of the action of heat on *anthrax* spores. The experimental values for this were obtained from experiments by Reichenbach (14 *b*).

TABLE V

Action of 0.6 Per Cent Phenol on Staphylococci (4b)

$a = 8.3$ $t_{\text{med.}} = 5.73$ min.

t	$e^{\frac{t_{\text{med.}} - t}{a}}$	Killed
min.		*per cent*
0	1.99	0
1	1.77	6.9
3	1.39	14.3
4	1.23	21.8
5	1.092	41.8
6	0.974	55.4
7	0.858	67.2
8	0.761	79.8
9	0.674	83.4
10	0.598	87.2
12	0.470	96.3
15	0.328	98.2

(*b*) On the basis of the assumption that the resistance distribution follows the rule of probability, the mortality-time curve can be constructed with the aid of Equation 3. If the average resistance of a group of bacteria is taken equal to 1 and the actual resistance of individual organisms is assumed to vary between $\pm\infty$ according to Gauss' probability rule with the precision 1, the probability p that all the organisms possess the resistance r can be obtained from Gauss' probability curve.[4] The probability that organisms in p fraction of the bacteria possess a resistance equal to or less than r is then 1. Thus, the probabilities will be the measure of the percentage of organisms killed and to each probability there will belong a resistance r obtainable from Gauss' curve. If this value is substituted in Equation 2, where h is expressed in multiples of $r_{\text{med.}}$ (that is, it is measured arbitrarily in units of $r_{\text{med.}}$) and if $a = 1$, the time can be calculated in units (multiples) of a. It is now possible to plot the probabilities (that is, percentage mortality) against $h' - r'$ (analogous to plotting against negative resistance) and against ln $(h' - r')$ (which

---

[3] The existence of this systematic error was pointed out by Yule (31). It must be emphasized, however, that it does not account quantitatively for the deviation of the mortality-time curves from the normal binomial distribution curves; *e.g.*, it could not account for the fact that a certain number of organisms survive in a solution having a definite concentration of a disinfecting agent.

[4] The table given in Czuber's Wahrscheinlichkeitsrechnung (Leipsic and Berlin, G. B. Teubner, 3rd edition, 1914) was used to obtain the values for this purpose.

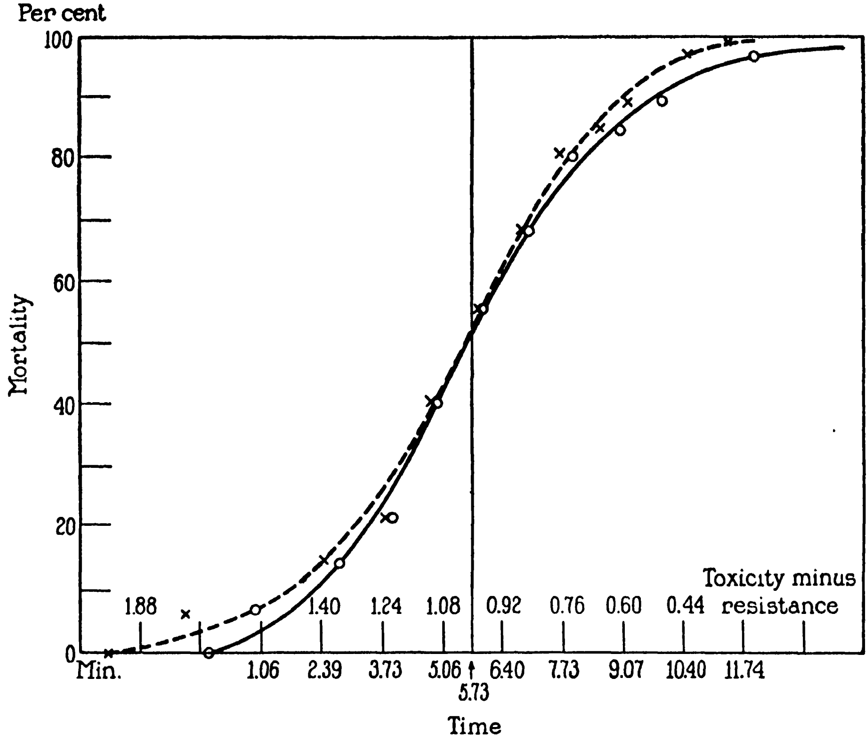

FIG. 4. Curves showing that asymmetrical mortality-time curve (———) obtained from an experiment on 0.6 per cent phenol with staphylococci (4b) becomes symmetrical if the mortality is plotted against $e^{\frac{t_{\text{med.}} - t}{a}}$ (- - - - -).

is proportional to the time) as was done with the experimental values. The points corresponding to $t_{\text{med.}}$ and $r_{\text{med.}}$ are again made to coincide on the abscissa and $h' - r'$ is allowed to decrease from the left to the right, whereas t is allowed to increase in the same direction.

Calculations were carried out in the manner described, first setting $h = 2.5$ and $r_{\text{med.}} = 1$ and then setting $h = 10$ and $r_{\text{med.}} = 1$. The results are given in Tables VII and VIII and illustrated in Figs. 6 and 7. Naturally the percentage

TABLE VI

Effect Produced on Anthrax Spores by a Temperature of 87°C. (14b)

$a = 16.1 \qquad t_{med.} = 11.2$ min.

t	$e^{\frac{t_{med.}-t}{a}}$	Spores killed
min.		*per cent*
1	1.882	0
2	1.765	9.54
5	1.471	39.04
10	1.078	53.14
15	0.791	74.24
20	0.580	87.14
30	0.312	95.47
45	0.123	99.32
63	0.040	99.90
90	0.007	102.14

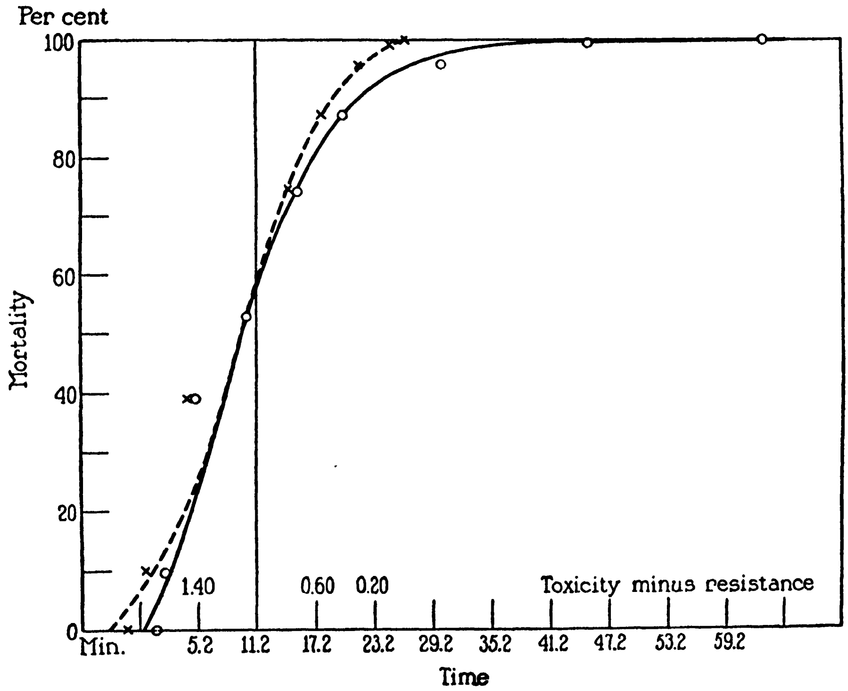

FIG. 5. Curves showing that asymmetrical mortality-time curve (———) obtained from an experiment on the effect of heat on anthrax spores (14*b*) becomes symmetrical if the mortality is plotted against $e^{\frac{t_{med.}-t}{a}}$ (- - - - -).

TABLE VII

Variation of the Time Calculated on the Basis That the Disinfecting Power is Constant and the Resistance Varies According to Probability

$h = 2.5 \qquad r_{\text{med.}} = 1.0$

	$\frac{h-r}{h-r_{\text{med.}}}$	$\ln\frac{h-r}{h-r_{\text{med.}}}$	Killed
			per cent
−0.40	1.933	0.662	2.38
−0.20	1.800	0.588	4.48
0.0	1.667	0.510	7.86
+0.20	1.533	0.427	12.89
+0.40	1.400	0.337	19.80
+0.60	1.269	0.237	28.58
+0.80	1.133	0.125	38.86
+1.00	1.000	0.000	50.00
+1.20	0.867	−0.143	61.25
+1.40	0.733	−0.311	71.42
+1.60	0.600	−0.509	80.2
+1.80	0.467	−0.763	87.11
+2.00	0.333	−1.100	92.14
+2.40	0.067	−2.705	97.62

TABLE VIII

Variation of the Time Calculated on the Basis That the Disinfecting Power is Constant and the Resistance Varies According to Probability

$h = 10 \qquad r_{\text{med.}} = 1.0$

r	$\frac{h-r}{h-r_{\text{med.}}}$	$\ln\frac{h-r}{h-r_{\text{med.}}}$	Killed
			per cent
−1.50	1.278	0.246	0.02
−1.00	1.222	0.204	0.23
−0.60	1.179	0.164	1.18
−0.40	1.156	0.143	2.38
−0.20	1.133	0.125	4.48
0.00	1.111	0.105	7.86
+0.20	1.089	0.085	12.89
+0.40	1.066	0.064	19.80
+0.60	1.046	0.045	28.58
+0.80	1.022	0.022	38.86
+1.00	1.000	0.000	50.00
+1.20	0.977	−0.023	61.13
+1.40	0.955	−0.046	71.42
+1.60	0.933	−0.069	80.20
+1.80	0.911	−0.093	87.11
+2.00	0.889	−0.118	92.14
+2.20	0.867	−0.143	95.52
+2.60	0.822	−0.196	98.82
+3.00	0.778	−0.251	99.76

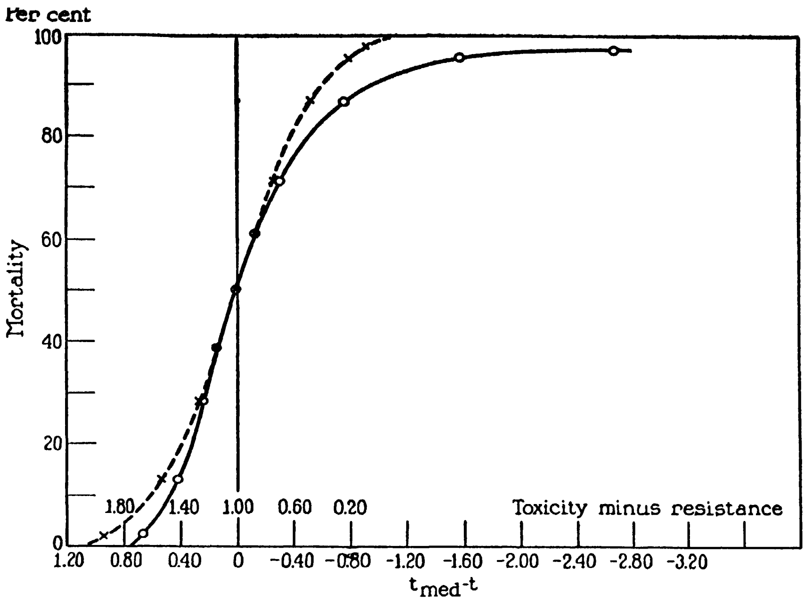

FIG. 6

FIGS. 6 and 7. Curves showing that symmetrical curves (- - - - -) corresponding to a variation of the resistance according to the probability law (Gauss) become asymmetrical if the probability (percentage mortality) is plotted against $\ln \frac{h-r}{h-r_{\text{med.}}}$ (———). This expression according to Equation 3 is proportional to time. In Fig. 6, $h = 2.5$, in Fig. 7, $h = 10$, and $r = a = 1$. Comparison of Figs. 6 and 7 shows that the asymmetry apparently decreases if h increases.

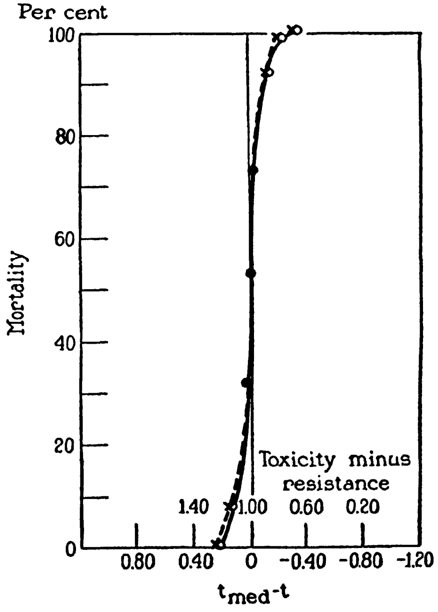

FIG. 7

mortality (probability of the resistance) plotted against $h' - r'$ shows in both cases perfect symmetry. The percentage plotted against $\ln(h' - r')$, that is, against values which according to Equation 3 are proportional to the time, results in an asymmetrical curve. The asymmetry is greater if $h = 2.5r_{\text{med.}}$ and less if $h = 10r_{\text{med.}}$, for the relative variation of the difference $h' - r'$ is smaller in the second case than in the first one. This is an effect, as was previously pointed out, which is known to persist with disinfection rate curves.

III. Variation of the Time of Survival with the Variation of the Toxicity If the Resistance and Temperature Are Kept Constant

(*a*) It is obvious that the relationship between time, resistance, and toxicity proposed above as an explanation for the asymmetry in the mortality curves of organisms exposed to a noxious agent must also be valid in cases where one is not dealing with a collective (statistical) phenomenon. It ought to be applicable in cases where one deals with a single organism or a group of organisms with ideally constant resistance and also in cases where one considers only one particular organism present in a group; *e.g.*, the one which possesses the median resistance or the maximum resistance. A great many experiments of the latter type are available in the literature. The application of Equation 2 to some of these cases will now be discussed. Experiments were selected so that the various noxious agents are very different in their chemical nature. The organisms used in these tests are also of very different nature but all are unicellular. If r is kept constant (r_h) and h varies, Equation 2 can be written in the following way:

$$(5) \quad a \ln \frac{h_1 - r_h}{h_n - r_h} = t_n - t_1$$

and solving for r_h one obtains—

$$(6) \quad r_h = \frac{h_1 - h_n e^{\frac{t_n - t_1}{a}}}{1 - e^{\frac{t_n - t_1}{a}}}$$

In order to apply Equation 5 to experimental values, h has to be expressed as a function of the concentration. While this function is not known so far, one can assume, for sufficiently small variations of h, that $\Delta h \sim \Delta c$, and

$$(7) \quad a \ln \frac{c_1 - r_c}{c_n - r_c} = t_n - t_1$$

where c means concentration.

Equation 7 is similar to one proposed by Ponder (27). The validity of Equation 7 is restricted to cases in which $h \sim c$, or to a range in which $\Delta h \sim \Delta c$. To calculate a one takes experimental values in which t_n is very large. In these experiments h_n is nearly equal to r for h approaches r if t_n approaches infinity. In practical cases it suffices to select experiments in which t_n equals a few hundred minutes in order to obtain an r value which differs from the correct one by but a few per cent at the most. a can then be calculated from two other experimental values according to the formula:

$$\frac{t_n - t_1}{\ln \dfrac{h_1 - r_h}{h_n - r_h}} = a$$

or if $h = c$

$$\frac{t_n - t_1}{\ln \dfrac{c_1 - r_c}{c_n - r_c}} = a$$

After having thus obtained a the remaining experimental values obtained for other concentrations of the toxic agent can be used to calculate r_h according to Equation 6, or if h can be taken as proportional to c, according to the analogous equation—

$$(8) \quad r_c = \frac{c_1 - c_n e^{\frac{t_n - t_1}{a}}}{1 - e^{\frac{t_n - t_1}{a}}}$$

The average of the r values thus obtained from a set of experiments can be used to recalculate a and the corrected values of r can be obtained, and so forth. If experimental values for long survival times are not available, a and r will first have to be estimated.

The median resistance of *Botrytis* spores against phenol was calculated in the manner described from experiments by Henderson Smith (30). It was found that the median resistance obtained from experiments with different phenol concentrations is, as expected, fairly constant. The survival time (*cf.* Table IX) was calculated with the aid of the average of the median resistance. The agreement between the calculated and experimental values is satisfactory with the exception of the value corresponding to the highest concentration and the shortest survival time.

TABLE IX

Action of Phenol on Botrytis Spores (30)

Calculation of the average resistance and of the time according to Equations 7 and 8.

$a = 91$

c	$t_{\text{med.}}$ (found)	t (calculated)	$r_{\text{med.}}$ (from 1 and n)
per cent	*min.*	*min.*	
0.4	92.9		
0.5	49.91	49.6	0.232
0.6	13.57	20.1	0.256
0.7	2.92	−1.74	0.222
			Average $r_{\text{med.}}$ = 0.237

Concerning the relation between concentration and disinfecting power, it seemed justifiable to assume that the first step of the process is the fixation of the agent on, or its entrance into, the cell. Since the cell represents a heterogeneous phase and since the experiments suggested that the accumulation of the agent in the cell is not proportional to the concentration, as it ought to be if the process follows the laws prevailing in solutions, it seemed proper to consider the possibility that the accumulation of the agent is due to its adsorption by the cell. If this were really the case, toxicity might be proportional to the adsorbed amount of the toxic agent throughout a fairly large concentration range. It was assumed that the function describing the relation between the toxicity and concentration, is similar to the well known type of function which describes the relation between the concentration and the amount adsorbed by a heterogeneous phase.

According to Langmuir (32), the amount (A) adsorbed by a square centimeter of a substance is

$$A = \frac{K_1 K_2 c}{K_2 c + 1}$$

If h is proportional to A it should also be proportional to $\frac{c}{c+\gamma}$ where $\gamma = \frac{1}{K_2}$. It is obvious that if γ is sufficiently large, or if the variation of the concentration is sufficiently small $\Delta h \sim \Delta c$ and thus for a small range Equation 5 can be used. However, by putting $h = \frac{c}{c+\gamma}$ in Equation 5 one obtains Equation 9 which has a much wider range of applicability as can be seen from the examples given below.

$$(9) \quad \frac{\dfrac{c_1}{c_1+\gamma} - r_h}{\dfrac{c_n}{c_n+\gamma} - r_h} = e^{\frac{t_n - t_1}{a}}$$

and

$$(10) \quad r_h = \frac{\dfrac{c_1}{c_1+\gamma} - \dfrac{c_n}{c_n+\gamma}\, e^{\frac{t_n-t_1}{a}}}{1 - e^{\frac{t_n-t_1}{a}}}$$

γ could be calculated from the adsorption isotherm of a toxic agent onto a given organism, if this were known. These isotherms have not been determined so far, hence γ must be estimated.

In Table X and Fig. 8, data are presented which were calculated from experiments by Chick (4*a*) and by Watson (33) on the action of phenol on *B. paratyphosus*. Since the range of concentration used in this set of experiments was small, both methods of calculation (that according to Equations 9 and 10 and that according to Equations 7 and 8) yield equally satisfactory agreement with the experiments.

Experiments by Chick (4*a*) on the action of silver nitrate on *B. paratyphosus* are given in Table XI and Fig. 9. The time and the resistance were calculated according to Equations 9 and 10. In spite of the fact that the range of concentration used in these experiments is very large, the agreement between calculated and observed values is satisfactory. Calculations on the basis of Equation 7 did not give constant values for the resistance and if the extrapolated threshold concentration was taken as equal to the resistance, the calculated times showed a considerable systematic deviation from the experimental values.

In the case of the action of mercuric chloride on *B. paratyphosus*, according to experiments by Chick (4*a*), experimental values and calculated values agree

TABLE X

Action of Phenol on B. paratyphosus (*33*)

Calculation of the time according to Equations 7 and 9, and of the resistance according to Equations 8 and 10.

Disinfecting power taken proportional to concentration (t_c and r_c).

Disinfecting power taken proportional to the adsorbed amount (t_h and r_h).

$a = 150$ $\gamma = 0.5$ $r_c = 0.498$ $r_h = 0.499$

c	$k_n = \frac{c_n}{c_n + \gamma}$	t (found)	t_c (calculated)	t_h (calculated)	r_c	r_h
per cent		*min.*	*min.*	*min.*		
0.800	0.615	45				
0.750	0.600	75	72	65	0.523	0.529
0.700	0.583	105	105	93	0.474	0.506
0.650	0.565	125	148	129	0.298	0.443
0.600	0.545	225	208	184	0.548	0.523
0.550	0.523	440	309	281	0.534	0.517
0.500	0.500	690	797	760	0.489	0.494

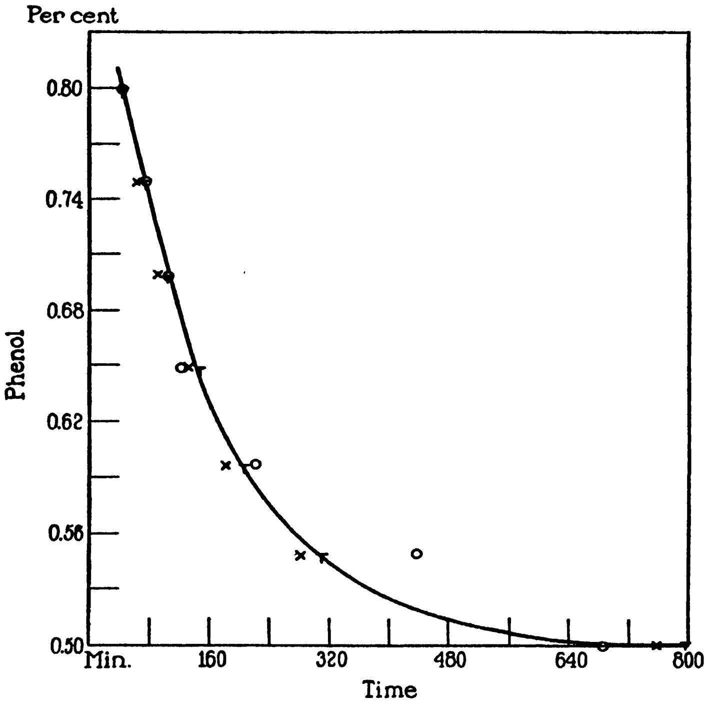

FIG. 8. Curve showing the relation between concentration and time of survival for the case of phenol and *B. paratyphosus* (33).

O = experimental values.

× = calculated values; disinfecting power taken as proportional to the concentration. Equation 7, $a = 150$ $r_c = 0.498$.

T = calculated values; disinfecting power taken as proportional to the adsorbed amount. Equation 9, $\gamma = 0.5$ $a = 150$ $r_h = 0.499$.

TABLE XI

Action of Silver Nitrate on B. paratyphosus (4a)

Calculation of the time according to Equation 9, and of the resistance according to Equation 10.

Disinfecting power taken proportional to the adsorbed amount.

$a = 70 \qquad \gamma = 0.0001 \qquad r_h = 0.400$

	$k_n = \frac{c_n}{c_n + \gamma}$	t (found)	t_h (calculated)	r_h
per cent		*min.*	*min.*	
0.085	0.9988	0.75		
0.017	0.9941	1.5	1.3	0.56
0.0085	0.9883	2.5	2.0	0.75
0.0017	0.9444	6.5	7.4	0.43
0.00085	0.895	22.5	14.0	0.75
0.00017	0.629	56.0	68.1	0.41
0.000085	0.459	140.0	163.2	0.43

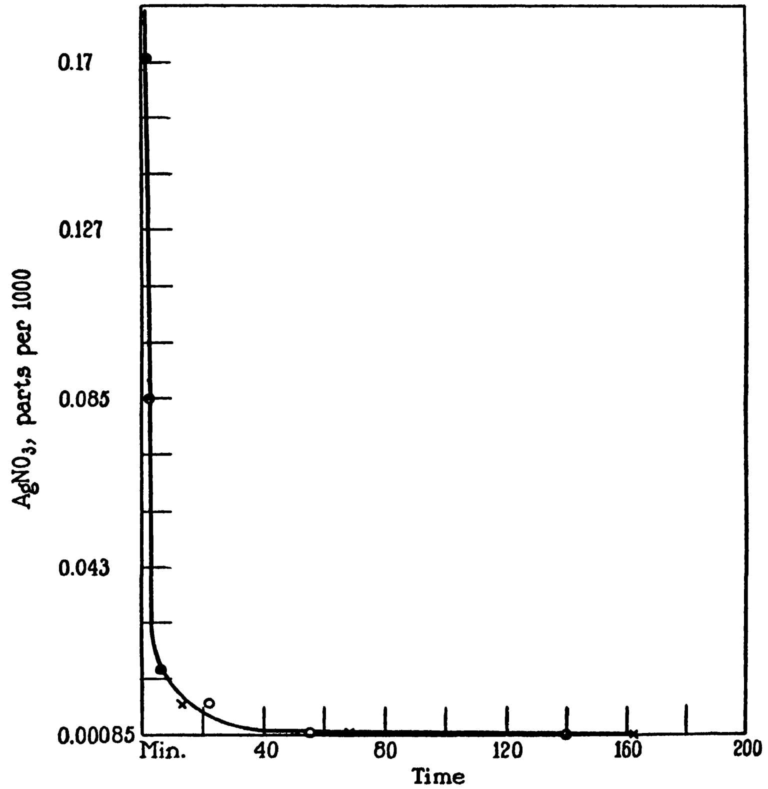

Fig. 9. Curve showing the relation between concentration and time of survival for the case of $AgNO_3$ and *B. paratyphosus* (4*a*).

○ = experimental values.

× = calculated values (Equation 9). $\gamma = 0.0001 \qquad a = 70 \qquad r_h = 0.400$.

only if one assumes that the toxicity of mercuric chloride is a function of the mercury ions present in the solution. The variation of the concentration of mercury ions remains small even though the variation of the concentration of the mercuric chloride is considerable, hence calculations according to Equation 9 and Equation 7 yield equally satisfactory results. For the ionization of mercuric chloride the values of Kahlenberg (34) were taken as quoted by Chick (20). (*Cf.* Table XII and Fig. 10.)

Measurements made by Gregerson (35) on the action of phenol on staphylococci cover a large range of concentration. Neither values calculated according to

TABLE XII

Action of Mercuric Chloride on B. paratyphosus (4a)

Calculation of the time according to Equations 7 and 9, and of the resistance according to Equations 8 and 10.

The disinfecting power taken as proportional to the Hg^{++} concentration (t_c and r_c).

The disinfecting power taken as proportional to the adsorbed amount of Hg^{++} (t_h and r_h).

$a = 38 \qquad \gamma = 30 \qquad r_c = 16.4 \qquad r_h = 0.353$

c = concentration of Hg^{++}; figures are proportional to concentration of Hg^{++}.

c	$h_n = \frac{c_n}{c_n + \gamma}$	t (found)	t_c (calculated)	t_h (calculated)	r_c	r_h
		min.	*min.*	*min.*		
63	0.678	1.5				
57.5	0.657	7	6	4	21.4	0.528
42.5	0.586	13	24	14	−45.3	0.169
37	0.552	10 (?)	33	20	29.9*	0.540*
23	0.433	65	76	55	18.7	0.396
16.5	0.354	230	236	222	16.4	0.352

* Calculated from values 2 and 4.

Equation 7, nor those calculated according to the assumption that the toxicity is proportional to the adsorbed amount of phenol (Equation 9) fit the experimental curves. The deviation, which is comparatively small in the latter case, can tentatively be explained by the fact that in this case at high concentrations the toxicity of the phenol is not strictly proportional to the adsorbed amount because in the concentrations used (up to 3 per cent) hypertonicity of the solution due to the osmotic pressure of the phenol adds to the toxic effect. This explanation seems more probable than any assumption of the existence of a systematic error due to the fact that, if the time is very small, the error becomes very great for the time required to establish equilibrium through diffusion is then of the same order as the survival time, namely a few seconds. (*Cf.* Table XIII and Fig. 11.)

It is of interest to note that the agreement between experimental and calculated values is good in the case of silver nitrate and paratyphoid bacilli, although the range of the variation of the concentration is greater than in the case of phenol and staphylococci. The probable reason for this is that the osmotic effect of the highest concentration of silver nitrate is still negligible.

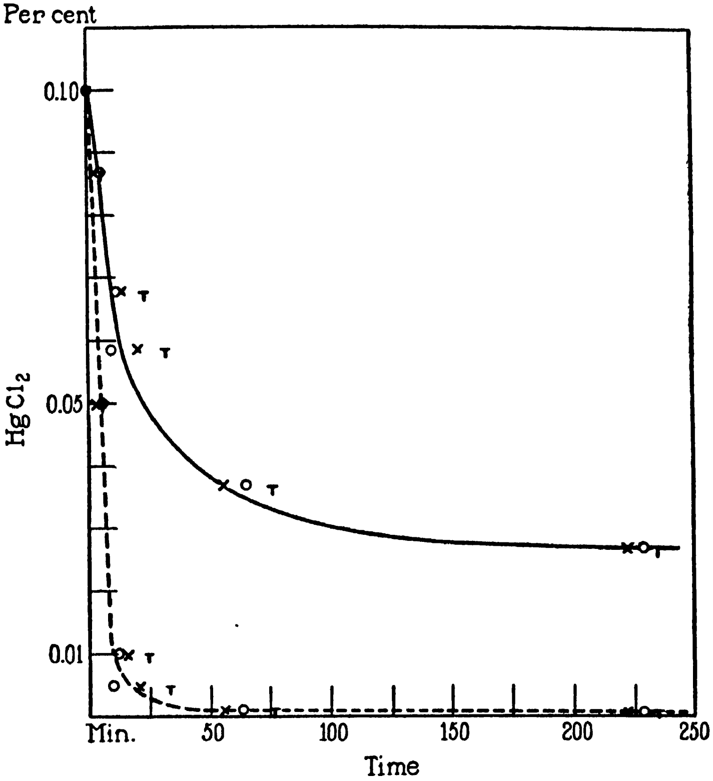

FIG. 10 Curves showing the relation between concentration of $HgCl_2$ and time of survival for the case of mercuric chloride and *B. paratyphosus* (4 *a*). Curve (- - - - -), time plotted against $HgCl_2$ (parts in 100). Curve (———), time plotted against numbers proportional to Hg^{++} concentration.

o = experimental values.

T = calculated values; disinfecting power taken as proportional to the concentration of Hg^{++} (Equation 7). $a = 38$ $r_c = 16.4$.

x = calculated values; disinfecting power taken as proportional to the adsorbed amount of Hg^{++} (Equation 9). $\gamma = 30$ $a = 38$ $r_k = 0.353$

For numbers proportional to the Hg^{++} concentration, see Table XIV.

It has been mentioned above that, in the case of hemolysis by saponin and

TABLE XIII

Action of Phenol on Staphylococci (*35*)

Calculation of the time according to Equations 7 and 9 and of the resistance according to Equations 8 and 10.

Disinfecting power taken as proportional to the concentration (t_c and r_c).

Disinfecting power taken as proportional to the adsorbed amount (t_h and r_h).

$a = 50 \qquad \gamma = 0.1 \qquad r_c = 0.490 \qquad r_h = 0.8330$

c	$k_n = \frac{c_n}{c_n + \gamma}$	t (found)	t_c (calculated)	t_h (calculated)	r_c	r_h
per cent		*min.*	*min.*	*min.*		
3.0	0.9677	0.187				
2.5	0.9615	0.375	11.3	2.5	−133	0.73
2.0	0.9523	1.0	25.6	6.3	−38	0.22
1.75	0.946	1.5	34.7	9.0	−27	0.31
1.50	0.937	3.0	45.8	12.8	−6.7	0.66
1.25	0.926	8.0	60.0	18.8	−1.14	0.82
1.00	0.909	20.0	79.8	28.8	0.077	0.84
0.875	0.897	30.0	93.9	37.4	0.31	0.84
0.750	0.882	75.0	113.8	50.7	0.67	0.87
0.625	0.861	90.0	146	78.7	0.27	0.80
0.500	0.8333	300	277	305	0.50	0.83

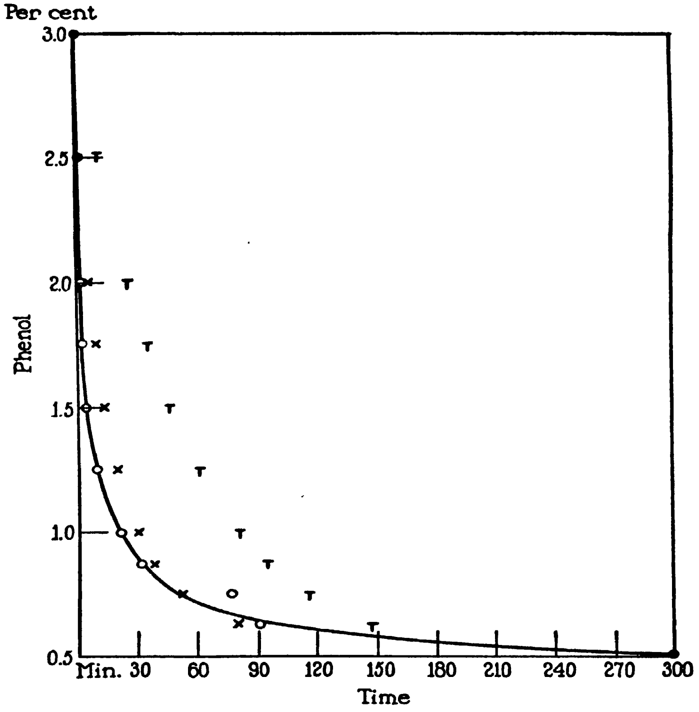

FIG. 11. Curve showing the relation between concentration and time for the case of phenol and staphylococci (35).

o = experimental values.

T = calculated values, disinfecting power taken as proportional to the concentration. (Equation 7). $a = 50$ $r_c = 0.490$.

× = calculated values; disinfecting power taken as proportional to the adsorbed amount (Equation 9). $\gamma = 0.1$ $a = 50$ $r_h = 0.8330$.

other agents, Ponder (27, 28)[5] has derived equations which he has tested on exact measurements of his own. It was of interest to see whether or not Equations 7 and 9 would give satisfactory agreement with the experimental values in this case also. Moreover, to test the equation on Ponder's measurements was interesting, because the material used in his experiments is so very different from the microorganisms and disinfecting agents which have been discussed and also because Ponder and Yeager's (28) measurements were much more exact than those made on disinfectants. In Table XIV the results of the calculations are compared with the experimental data. Values calculated according to Equation 9 gave very good agreement with the experimental data, whereas values calculated according to Equation 7 showed a systematic deviation from the experiments and hence were omitted from the table. Equation 7 was not applicable, although the range in which the concentration varied was comparatively small. This is probably due to the fact that saponin is high in capillary activity, hence the adsorption is not proportional to the concentration even at low concentration.[6]

TABLE XIV

Action of Saponin on Mammalian Red Cells (28)

Calculation of the average time according to Equation 9 and of the resistance according to Equation 10.

$a = 4 \qquad \gamma = 0.005$

c	$h_n = \frac{c_n}{c_n + \gamma}$	$t_{med.}$ (found)	$t_{med.}$ (calculated)	$r_{med.}$ (from 1 and n)
per cent		*min.*	*min.*	
0.00125	0.713	7.91		
0.00143	0.741	5.50	5.51	0.681
0.00167	0.769	4.17	4.02	0.677
0.00200	0.800	2.87	2.83	0.680
0.00250	0.834	1.73	1.83	0.680
0.00333	0.870	0.95	1.00	0.679
				Average $r_{med.}$ = 0.679

(*b*) In analogy to the theoretical mortality-time curves, one can construct a set of theoretical concentration-time curves by measuring the time in units of a and the toxicity in units of r (the constant resistance). If γ is very great, h becomes proportional to the concentration. The shape of this curve together with that calculated for a moderate γ is given in Fig. 12 and the corresponding calculated values in Table XV.

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[5] I am indebted to Dr. Ponder for calling my attention to his interesting papers and for discussing them with me.

[6] $t_{med.}$ was interpolated in this case.

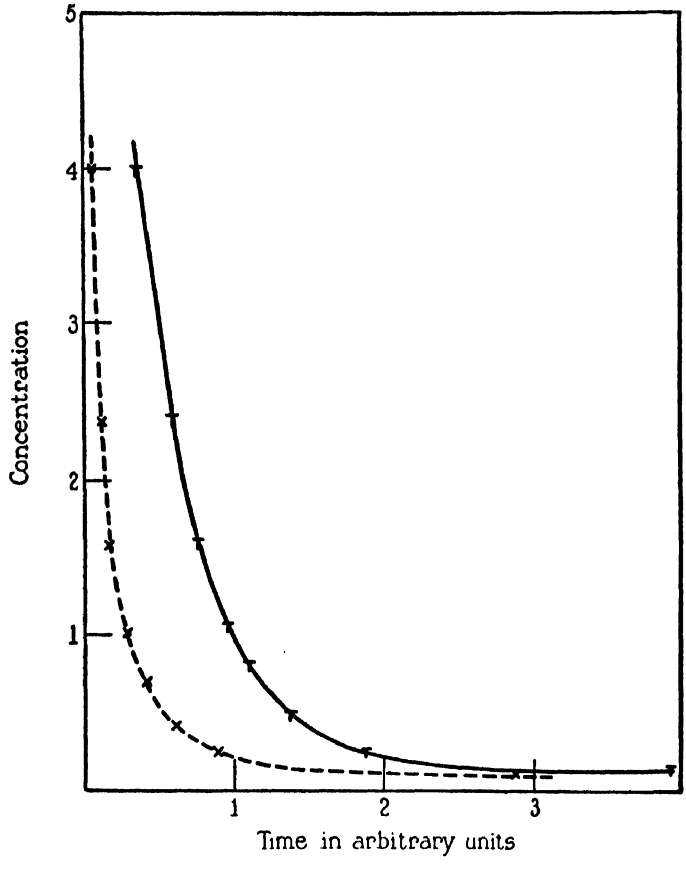

FIG. 12. Curve showing the theoretical relations between time and disinfecting power, the latter measured in multiples of the constant resistance, the time being measured in units of a $(a = 1)$.

———T the disinfecting power taken as proportional to the concentration (Equation 7).

- - - - - X the disinfecting power taken as proportional to the adsorbed amount (Equation 9).

The shapes of the concentration-time curves are different for different organisms and toxic agents and are characteristic for a given organism and toxic agent. The shape is determined by the constants, a, γ, and r. γ determines mainly the slope of the vertical branch of the curve, while r determines the position of the horizontal branch.

It is difficult to test Equation 2 for cases in which the toxic agent is injected into higher animals, for the relation between toxicity (h) as defined in this paper and the dose is not known. It seems to be hazardous to make any assumption about this relation since the conditions are certainly very complicated. Mechanical distribution in the blood stream, diffusion through the tissue, specific site of noxious action, excretion, and chemical transformation should be taken into consideration. The shape of the dose-time curves must, however, be similar to that of the curves given in Fig. 12.

TABLE XV

Relation between Time and Disinfecting Power

Disinfecting power taken as proportional to concentration (c).

Disinfecting power taken as proportional to the adsorbed amount (h).

$\gamma = 1 \qquad a = 1 \qquad r_c = 0.110 \qquad r_h = 0.099$

c	$\log\frac{c_1 - r_c}{c_n - r_c}$	$h_n = \frac{c_n}{c_n + \gamma}$	$\log\frac{h_1 - r_h}{h_n - r_h}$
9		0.9	
4	0.358	0.8	0.0577
2.33	0.602	0.7	0.125
1.50	0.807	0.6	0.204
1.00	1.000	0.5	0.300
0.667	1.202	0.4	0.425
0.428	1.447	0.3	0.600
0.250	1.803	0.2	0.900
0.111	3.95	0.1	2.904

IV. Variation of Percentage Mortality (Probability of Resistance) with Variation of the Concentration, If the Time and Temperature Are Constant

It appears from Equation 2 that if the time is constant, the difference between toxicity and resistance must remain constant, that is, changes in the toxicity involve equal changes in the resistance. If $t_n = t_1$ Equation 3 can be written as—

$$(11) \qquad \frac{h_1 - r_1}{h_n - r_n} = 1 \quad \text{and} \quad h_1 - h_n = r_1 - r_n$$

or

$$\frac{c_1}{c_1 + \gamma} - \frac{c_n}{c_n + \gamma} = r_1 - r_n$$

Since in this case the percentage mortality is the expression of the probability that all the organisms present have a resistance equal to or smaller than the toxic power, the mortality-resistance curve is a symmetrical variation curve (Gauss' probability curve). Consequently the percentage mortality plotted against the toxicity must also result in a symmetrical variation curve. This, however, does not necessarily hold for the mortality-concentration curves, for as a rule the concentration is not a linear function of the toxicity. If the variation of the resistance in a group of organisms is small, the difference between the concentration of the toxic agent which kills a detectable number of the organisms and that which kills all of them, will be small and in the corresponding range of concentration the latter will be proportional to the toxicity. In this case the mortality-concentration curve should be symmetrical. If the variation of the resistance is great, the corresponding variation of the concentration will also be great and the toxicity will not be proportional to the concentration. In this case the mortality-concentration curve should be asymmetrical.

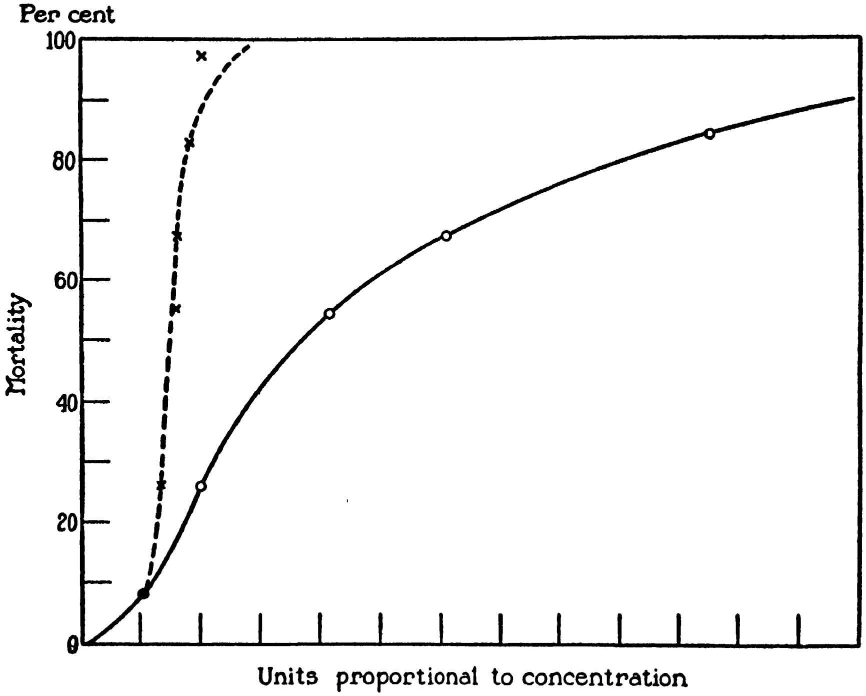

FIG. 13. Curves showing the variation of the percentage of *Macrosporium* killed by H_2S at a certain time (36). The asymmetrical mortality-concentration curve (-Θ-Θ-) tends to become more symmetrical if the mortality is plotted against the adsorbed amount $\sim \frac{c}{c + \gamma}$ (×···×··).

Most of the mortality-concentration curves given in the literature are symmetrical. The asymmetry is certainly not great enough to offer an opportunity to test the validity of Equation 11. McCallan and Wilcoxon (36) have observed mortality-concentration curves which were asymmetrical. In this case, if the percentage mortality is plotted against $\frac{c}{c+\gamma}$ (where γ is estimated) the asymmetry becomes less conspicuous (*cf.* Fig. 13).[7]

V. Variation of the Time with the Variation of the Temperature If the Concentration and Resistance are Constant

The effect of temperature on the survival time of organisms exposed to a toxic agent has been studied by Krönig and Paul (1), Chick (4), Reichel (19), and others. It was found more or less empirically that the time is an exponential function of the temperature analogous to that proposed by Arrhenius for the temperature coefficient of the velocity constants of chemical reactions. In Equation 2, a is the only constant which necessarily varies with the temperature. The reciprocal of a has the character of a velocity constant and thus it is not surprising that it follows a law similar to that of the velocity constant of chemical reactions.

For a temperature T and T_0

$$\frac{h_{T_0} - r_{T_0}}{h_T - r_T} = e^{\frac{t+K}{a} - \frac{t_0+K_0}{a_0}}$$

Taking into consideration that $h_T = h_{T_0}$ and $r_T = r_{T_0}$ and assuming that $\frac{K}{a} = \frac{K_0}{a_0}$ one derives $\frac{t}{a} = \frac{t_0}{a_0}$; and if $a = a_0 e^{(T_0-T)\alpha}$

$$(12) \quad t = t_0 e^{(T_0-T)\alpha}$$

which is the equation used by Chick, Reichel, and others.

Table XVI and Fig. 14 show that in the case of the action of 1 per cent phenol on *B. paratyphosus* at different temperatures (Chick, 4*a*) the values of α in Equation 12 are fairly constant. The time calculated with the average of α is in satisfactory agreement with the experimental data. According to these results, if all the conditions except the temperature are kept constant, the survival time is determined by Equation 12.

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[7] After completion of the manuscript it was noted that unpublished experiments by A. C. White are quoted in the recently issued monograph by Clark (21). In these, mortality-dose curves are compared with mortality curves plotted against a non-linear function of the dose. The function used is similar to an adsorption isotherm. It is noted that, whereas the distribution of survivals is normal if plotted against this function of the concentration, it becomes asymmetrical when it is plotted against the concentration. This is a result which is in full agreement with the theory discussed in this paper.—At the time of proof-reading, appearance is noted of a theoretical consideration of the relationship of dose to time (Gehlen, W., *Arch. Exp. Path. u. Pharm.*, 1933, **171,** 541).

TABLE XVI

Time of Survival of B. paratyphosus Exposed to 1.0 Per Cent Phenol at Different Temperatures (4a)

Time calculated according to Equation 12.

Temperature (T)	Time (t) (found)	α	Time (calculated)
degrees absolute	*min.*		*min.*
273.0	1050	17,830	1235
279.0	226	17,310	296
288.8	23.5	16,520	31.6
289.5	27.0	18,210	27.3
294.3	9.0	17,430	9.8
300.5	3.5	22,190	3.0
303.5	1.5	Average = $\bar{\alpha}$ = 18,248	

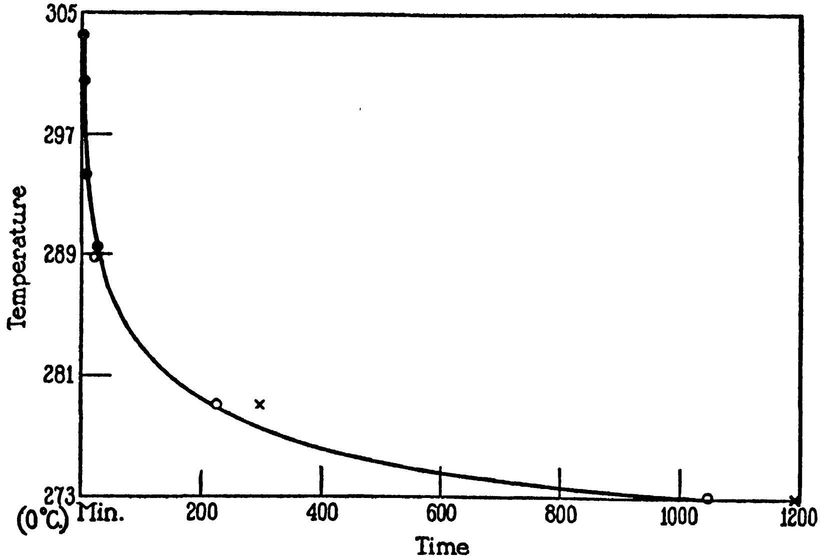

FIG. 14. Curve showing the relation between temperature and time of survival for the case of the constant disinfecting power of 1 per cent phenol on *B. paratyphosus* at different temperatures (4*a*).

o = experimental values.

× = calculated values on the basis of Equation 12 (*cf.* Table XVI).

TABLE XVII

The Effect of Temperature on the Time of Survival of Anthrax Spores (37)
Time calculated according to Equation 12.

Temperature (T)	Time (t) (found)	α	a_n	Time (calculated)
degrees absolute	*min.*			*min.*
360.0	90		16.1*	
363.4	14.7	70,000	4.39	24.5
365.7	8.7	54,041	1.85	10.3
367.2	5.0	53,200	1.048	5.9
370.45	3.16	42,848	0.316	1.8
374.2	1.14	41,400	0.083	0.5
378.3	0.43	39,791	0.019	0.11
		Average = $\bar{\alpha}$ = 50,213		

* This value was taken from Reichenbach's experiment (*cf.* Table VI, Fig. 5).

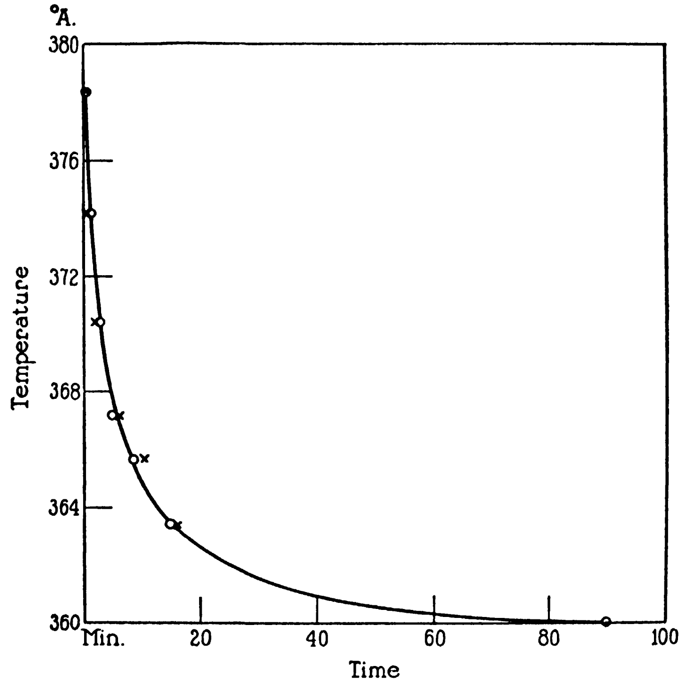

FIG. 15. Curve showing the relation between temperature and time of survival for the case of the effect of heat on anthrax spores (37).

o = experimental values.

× = calculated values on the basis of the assumption that the disinfecting power due to heat is constant (Equation 12). *Cf.* Table XVII.

The killing of microorganisms at high temperatures is usually considered as a result of killing power due to heat. While it is questionable whether h, the killing power, is changed or not if all conditions except the temperature are kept constant, it is conceivable that the noxious action due to heat is a constant and that it is merely the constant a which decreases when the temperature increases and thus the time of survival is shortened.

Survival times of anthrax spores have been calculated from experiments by Ballner (37) by calculating first α for different time intervals and then the time with the average of α. Values for α for different time intervals show a distinct trend possibly indicative of the fact that the disinfecting power is itself a function of the temperature or that the assumption that $\frac{K}{a} = \frac{K_0}{a_0}$ is not correct in this case. The deviation might, however, also be caused by a systematic error due to lack of precision in the determination of the time at which the exposure to this temperature has been discontinued. (*Cf.* Table XVII and Fig. 15.)

DISCUSSION

In considering the agreement between experimental and theoretical values it must be remembered that disinfection experiments are subject to a considerable experimental error, the sources of which have been discussed extensively before. Added to this is the fact that the theoretical time values imply that the probability of the resistance under consideration having the assumed value is 1. This probability can only be approached but never attained. In other words the theory of probability ascribes for the *n*th bacterium of a group arranged according to their resistance, a definite resistance. Whether, in a certain determination, the *n*th organism will actually have this resistance, or a different one, cannot be predicted. It will depend on the errors of the method which have themselves the character of probable errors and cannot be distinguished from the actual variations of the resistance which also follow the probability rule. The error will be especially great when the concentration approaches the threshold value of the resistance, for a very small deviation in the percentage mortality will produce a considerable variation in the time. However, the slope of the curve at this point is practically zero and thus the calculated and experimental points will still fall on the same curve. This is the reason why the agreement appears to be much better according to the curves given in this paper than according to the tables.

It must also remain undecided whether the variation of the resistance in a group of organisms is actually due to the fact that the resistances of individual organisms are different and distributed according to the probability rule or due to the fact that the process of dying lacks precision and causes the time of survival to be different although the resistance of the organisms is the same. If the latter were the case, and if an experiment could be repeated with one and the same organism several times, it should lead each time to a different result and the distribution of the observed times should be the same as that which can be observed in one experiment with a group of organisms (this could be achieved in a case in which the noxious action is reversible; *i.e.*, narcosis).

Any noxious agent acting on a unicellular organism can be characterized by the value of three constants: (1) the resistance, which is the threshold value of the toxic power which a certain organism can stand without a lethal effect,[8] (2) the constant a which determines the time which is required for the completion of the destruction of the organism if the difference between the toxicity and the resistance is unity. Thus, two agents which kill an organism in the same concentration at the same time are not necessarily identical. Their apparently similar behavior might have different causes. Against the first agent, the organism in question might have a very low resistance but the value of the constant a_1 might be very great. Against the second agent, the resistance of the organism might be very high but a_2 might be very small. If the constant a is very large it is customary to speak about a slow disinfecting agent. If a large a is combined with a large variation of the resistance, most of the organisms will be killed provided that the observation is extended for a sufficiently long time, but some might survive. They start to propagate but most of the newly formed organisms, being exposed, will also succumb. A stationary state may develop. Agents having this effect on bacterial suspensions are called growth-inhibiting agents. It seems that there is no fundamental difference between them and disinfecting agents. Although in general (*e.g.*, for higher organisms) there is no way to measure the toxicity and thus no way to measure the resistance, in the case of unicellular organisms it seems that the toxicity is a function of the concentration which is similar to an adsorption isotherm. Since thus the relation between toxicity and concentration is known, the toxicity and resistance can be expressed in terms of concentration. (3) In order to be able to calculate the toxicity from the concentration even in arbitrary units the knowledge of a third constant γ is required. The value γ indicates whether at the concentration in question the organism is nearly saturated with the agent or whether around this concentration the agent is bound proportionally to the concentration. Thus, two agents which would kill a certain organism in the same concentration and in the same time need not be identical even though the constant a is the same, for the one agent might be a strongly noxious agent but scarcely bound at all, whereas the other might be a weakly noxious agent but very well adsorbed.

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[8] The damaging effect of sublethal concentrations of certain agents seems to play an important rôle in the action of the so called chemotherapeutic agents.

These examples illustrate the interesting possibilities which are offered by the application of Equation 2 to the characterization and test of toxic agents. A discussion of the practical application together with criticism of present methods based on the viewpoints presented in this paper will appear later.

It is to be expected that the given relationship can be applied to various biological and pharmacological phenomena, the common feature of all of which is the existence of a time factor and of a threshold value.

SUMMARY

1. A relation between toxicity, resistance, and time of survival has been derived on the basis of the assumption that the time is a function of a parameter which is the difference between the toxicity and the resistance. Toxicity and resistance act like forces which can maintain an equilibrium-like (or stationary) state. If the equilibrium is upset, the time at which the event (death) occurs is proportional to the logarithm of the difference between toxicity and resistance.

2. It was found that if values proportional to the resistance are calculated with the proposed equation and the percentage mortality plotted against them (instead of against the time as is usual) symmetrical curves are obtained even though the corresponding mortality-time curves are asymmetrical. Assuming that the resistance varies

like an error, that is, according to probability rules, theoretical mortality-time curves, similar to the experimental curves, can be constructed from the proposed equations.

3. In the case of a toxic agent acting on a unicellular organism suspended in solution, the toxicity is proportional to the adsorbed amount of the agent, as calculated with the aid of the Langmuir equation. In small concentration ranges the toxicity can be taken as approximately proportional to the concentration.

4. The variation of the temperature affects mainly the constant a which is a function of the temperature similar to that of the velocity constant of a chemical reaction (Arrhenius' law).

5. The proposed equation has been tested in four different combinations of the variables, concentration, resistance, time, and temperature. The agreement with the experiments is satisfactory.

6. Any noxious agent acting on a unicellular organism may be characterized by three constants: r, the resistance, which is the threshold value at which the agent is still fatally toxic for the organism; a, the reciprocal of the rate constant determining the specific rate (that is, the time corresponding to a difference of 1 between the toxicity and the resistance); and finally the constant γ of the function representing the relation between toxicity and concentration.

The writer is indebted to Miss Alma J. Champlin for valuable assistance in the collection of experimental data, calculations, and preparation of the manuscript.

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THE KINETICS OF PENETRATION

VI. Some Factors Affecting Penetration

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INTRODUCTION

Although sea water contains very much more sodium than potassium the latter predominates in the cells of many marine organisms. Presumably this is because it penetrates the protoplasm more rapidly.[1] A like situation in models affords a favorable opportunity to study certain variables which may be important in living cells.

In these models[2] electrolytes pass from an outer aqueous layer A (Fig. 1) through a non-aqueous layer B (representing the protoplasm) into an inner aqueous layer C (which may be called "artificial sap"); the latter consists at the start of distilled water and CO_2 bubbles in it throughout the experiment.

All three layers are stirred mechanically but at the phase boundaries are unstirred layers ($A_B B_o B_i$, and C_B) in which the movement of electrolytes depends on diffusion. The layers B_o and B_i, in which diffusion is slowest,[3] regulate the process of penetration.

In these experiments A contained KOH and NaOH which had been previously shaken up with a non-aqueous mixture consisting of 70 per cent guaiacol + 30 per cent *p*-cresol (which will be called G. C. mixture). As a result KOH combined with the constituents of the non-aqueous phase to form organic salts which may be lumped together[4] and called collectively K*G*, the corresponding designation for the sodium salts being Na*G*. We therefore have to do with the penetration of K*G* and Na*G*.

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[1] Osterhout, W. J. V., *Biol. Rev.*, 1931, **6**, 369; *Ergebn. Physiol.*, 1933, **35**, 967.

[2] Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931–32, **15**, 667.

[3] See p. 448. Also Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529.

[4] This introduces no serious error since the behavior of the organic salts seems to be very similar. *Cf.* footnote 2.

In the preceding paper[5] it was assumed that the rate of penetration of potassium is proportional to the concentration gradient in the non-aqueous layer *B*. This gradient may be regarded as $K_o' - K_i'$, where K_o represents the concentration of undissociated K*G* in the outer surface of B_o, and K_i' that in the inner surface of B_i (the corresponding designations for sodium are Na_o' and Na_i').

On reaching *C*, K*G* and Na*G* come in contact with CO_2 and form $KHCO_3$ and $NaHCO_3$. This raises the osmotic pressure in *C* so that water enters from *A*. Eventually a steady state is reached in which water and salts enter in a fixed ratio and the volume of *C* increases while its composition remains constant. This appears to be analogous to what happens in living cells.

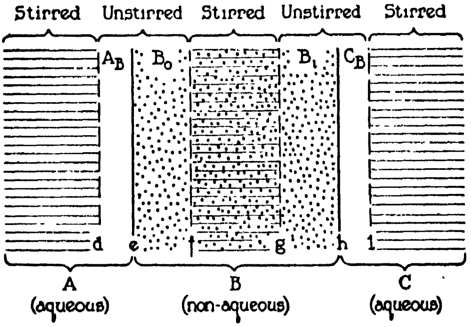

FIG. 1. Diagram of layers in the model. The aqueous phase *A* has an unstirred layer which is represented between *d* and *e* : from *e* to *f* is the corresponding unstirred layer in the non-aqueous phase *B*. Similar layers are present at the boundary between the non-aqueous phase *B* and the aqueous phase *C*.

Previous studies[6] indicate that the rate of entrance is proportional to $K_o' - K_i'$: it is also proportional to the diffusion constant. Hence we may write as an approximation

$$R_K = C_1 D_K (K_o - K_i)$$

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[5] Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529.

[6] This equation takes no account of the entrance of water. Regarding the significance of the "constants" see Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529.

Here R_K is the rate of entrance of moles of potassium into C and D_K the diffusion constant of undissociated KG in B. Using a corresponding notation for sodium we have

$$\frac{R_K}{R_{Na}} = \frac{C_1 D_K (K'_o - K'_i)}{C_2 D_{Na} (Na'_o - Na'_i)}$$

C_1 and C_2 are "constants"[6] which depend on the rate of stirring, the surface area, and shape of B, etc., and when they are the same for KG and NaG cancel out.

This equation permits only qualitative predictions because the ratio $(K'_o - K'_i) \div (Na'_o - Na'_i)$ changes during the process[7] and several disturbing factors intervene (p. 455).

Let us now consider K'_o and K'_i. We may make the usual assumption that on each side of the phase boundary there are very thin layers in approximate equilibrium with each other. Hence if the total concentrations of potassium (KG + KOH + $KHCO_3$) in the aqueous surface layers be K_o and K_i we may put

$$\frac{K'_o}{K_o} = S_{K_o} \quad \text{and} \quad \frac{K'_i}{K_i} = S_{K_i},$$

where S_{K_o} and S_{K_i} are the partition coefficients or absorption coefficients.[8]

Hence we may put

$$\frac{R_K}{R_{Na}} = \left(\frac{D_K}{D_{Na}}\right) \frac{S_{K_o} K_o - S_{K_i} K_i}{S_{Na_o} Na_o - S_{Na_i} Na_i}$$

In view of this a knowledge of diffusion constants and partition coefficients becomes desirable.

Diffusion Constants

When only KG is present the meaning of the diffusion constant D_K requires no comment, but when we add NaG it will affect the value of

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[7] The behavior of this ratio depends on the magnitudes of the partition coefficients as well as on their ratio. See p. 449.

[8] *Cf.* Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 125; 1931–32, **29**, 993, 1234; *J. Gen. Physiol.*, 1928–29, **12**, 147, 407.

D_K. The divergence will be small in guaiacol (in which K*G* and Na*G* are very weak electrolytes)[9] but may be larger in water: this latter, however, does not concern us since for our purposes the diffusion in water may be regarded as negligible. Similar reasoning applies to the diffusion constant D_{Na}.

Let us now consider the ratio $D_K \div D_{Na}$. In order to determine this the diffusion apparatus of Northrop and Anson[10] was used. The upper part was filled with G. C. mixture containing equal concentrations of K*G* and Na*G* which were allowed to diffuse into the lower chamber containing G. C. mixture. The result showed that D_K is so close to D_{Na} that for our purposes they may be regarded as equal.

Similar tests in which water was employed as the solvent showed that K*G* and Na*G* diffuse ten to eleven times as fast in water as in G. C. mixture (this result is due in part to the higher viscosity of the G. C. mixture[11]). Hence the diffusion through the unstirred aqueous layers A_B and C_B is so rapid that it may be neglected in the subsequent discussion. We need only consider the slow diffusion in the non-aqueous layers B_o and B_i.

Partition Coefficients

Evidently the diffusion constants, D_K and D_{Na}, are too similar to account for the experimental fact that potassium enters *C* much faster than sodium. It would seem that the partition coefficients must be responsible for this difference.

In order to clarify the rôle of the partition coefficients let us first consider an hypothetical system in which K*G* and Na*G* are very weak electrolytes in *A*, *B*, and *C*, and in which *C* contains no CO_2. Let the values of K_o and Na_o be constant at 0.05, the values[12] of S_{K_o} and S_{K_i} being 0.62, those of S_{Na_o} and S_{Na_i} being 0.29, and those of D_K and D_{Na}

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[9] This has been shown by a group of physical chemists whose results will shortly be published.

[10] Northrop, J. H., and Anson, M. L., *J. Gen. Physiol.*, 1928–29, **12,** 543.

[11] The viscosity of water at 30.73°C. in C. G. S. units is 0.007905 and that of guaiacol at 30°C. is 0.0445. *Cf.* Landolt, H., and Börnstein, R., Physikalisch-chemische Tabellen, Berlin, Julius Springer, 5th edition (Roth, W. A., and Scheel, K.), 1923, **1,** 135, and 1931, suppl. vol. **2,** 102.

[12] These vary with concentration but we may assume for purposes of calculation that S_{KG_i} is constant as well as S_{KG_o}.

being equal. A simple calculation[13] indicates that in the early part of the experiment potassium should enter C faster than sodium and that $K_i \div Na_i$ should be less than $S_{K_o} \div S_{Na_o}$. The experiments bear this out. The calculation is as follows:

Let us suppose that the unstirred layers are so thin that the concentration gradient in B is approximately linear and that C is a mere film, only a few molecules thick, and contains at the start only distilled water, the bubbling of CO_2 being omitted. Let us now consider a layer in B of the same thickness as C adjoining the interface: this will be called B_{is}. For convenience we suppose the total volume of each of these films to be 1 liter.

At the start of the experiment the concentration gradient of KG in B, which will be called G_K', is $0.031 - 0 = 0.031$, and the corresponding value for G'_{Na} is $0.0145 - 0 = 0.0145$. Hence $G_K' \div G_{Na}' = 2.14$. We may assume that during the first small increment of time 1.62 millimoles of KG reach B_{is} and distribute themselves so that 1.0 goes into C and 0.62 remains in B_{is}, giving a concentration of 0.00062 M in B_{is} and 0.001 M in C (the value of K_o' remains constant at 0.031 since K_o is constant).

Since at the start G'_{Na} is 0.0145 and G'_K is 0.031 we suppose that the amount of NaG moving across B in the first small increment of time is approximately $1.62 \, (0.0145 \div 0.031) = 0.756$ millimole: of this 0.17 will remain in B_{is} and 0.586 will pass into C (so that $S_{Na_i} = 0.17 \div 0.586 = 0.29$; this is the value previously stated). At the end of the first increment of time $G_K' = 0.031 - 0.00062 = 0.0304$ and $G_{Na}' = 0.0145 - 0.00017 = 0.0143$. Hence $G_K' \div G_{Na}' = 0.0304 \div 0.0143 = 2.1$ and $K_i \div Na_i = 0.001 \div 0.000586 = 1.7$.[14]

This calculation indicates that in the early part of the experiment the ratio $K_i \div Na_i$ will be greater than unity and that as time goes on it will approach unity since the concentrations in *C* will approach those in *A* (*i.e.* 0.05 M K*G* + 0.05 M Na*G*).

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[13] The significance of this method of calculation may seem doubtful, especially when we are not dealing with the earliest stages of the experiment, but it seems to be borne out by the results.

[14] It is of interest to note that the ratio $K_i \div Na_i$ in the early part of the experiment depends on the magnitudes of S_{KG_o} and S_{NaG_o} as well as on their ratio. Thus in the foregoing calculation we had $S_{KG_o} \div S_{NaG_o} = 0.62 \div 0.29 = 2.14$ and the value $K_i \div Na_i$ after the first increment of time was 1.7, but if we put $S_{KG_o} \div S_{NaG_o} = 6.2 \div 2.9 = 2.14$ we get a different result for $K_i \div Na_i$ since the value is 1.16. This may be shown as follows. Since G_K' and G_{Na}' are now ten times as great at the start we expect 16.2 millimoles of potassium and 7.56 of sodium to be moved. The concentrations in B_{is} are therefore KG = 0.0162 M and NaG = 0.00756 M. These will distribute so that $K_i' = 0.01395$ M, $K_i = 0.00225$ M, $Na_i' = 0.00562$ M, and $Na_i = 0.00194$ M. Hence $K_i \div Na_i = 0.00225 \div 0.00194 = 1.16$.

The actual model (with no CO_2 in *C*) differs from this hypothetical case since S_{K_i} is not constant and is not equal to S_{K_o}: this applies also to sodium. Furthermore K*G* and Na*G* are strong electrolytes in *A* and *C*[1] (though weak electrolytes in *B*) but this merely means that the diffusion of molecules of K*G* and Na*G* in *C* is replaced by the diffusion of the ions K^+, Na^+, and G^-, thereby changing somewhat the diffusion constants.

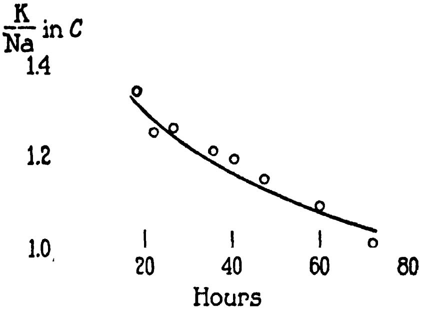

FIG. 2. Ratios of potassium to sodium in the artificial sap in *C* (Experiment 112). Model with 0.05 M K*G* + 0.05 M Na*G* in *A*, G. C. mixture in *B*, and distilled water in *C* (no CO_2). The ratio of partition coefficients is $S_{KG_o} \div S_{NaG_o} = 2.14$. Toward the end of the experiment the ratio approaches unity, as would be expected. In the first part of the curve the experimental errors are larger because the concentrations of sodium and potassium in *C* are relatively low.

Hence we expect that at the start potassium will enter *C* more rapidly than sodium and that both will reach the same concentration at equilibrium. This was found to be the case in earlier experiments (*e.g.* in Experiment 66 reported in a previous paper[15]). The ratios found in a later experiment (Experiment 112) are shown in Fig. 2 (in this, as in the other figures, the curves are drawn free-hand to give an approximate fit). In this case *A* contained 0.05 M K*G* + 0.05 M Na*G* and the ratio $S_{KG_o} \div S_{NaG_o}$ was 2.14 (see page 452).

Model I was employed and a steady flow was maintained in *A* which kept its composition approximately constant: *B* contained G. C. mixture and *C* contained distilled water (no CO_2 bubbling), stirred with a stream of air. The volume of C at the start was 150 cc. The temperature was 21° ± 2°C. The concentration of potassium + sodium in C was determined by titration with standard acid and of sodium by the method previously described,[2] potassium being determined by difference.

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[15] Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931–32, **15**, 667, Fig. 6.

In most of our experiments CO_2 was present in C and in consequence KG and NaG reacted to form $KHCO_3$ and $NaHCO_3$ as soon as they reached the surface of C.[16] Potassium and sodium then diffused through C_B in the form of bicarbonates. But since diffusion in this layer is very rapid as compared with that in B it may be left out of account. (An additional effect of the reaction with CO_2 is a great reduction of the ionic activity products (K)(G) and (Na)(G) in C.)

Before discussing such experiments it is desirable to consider more fully the equation for penetration. In doing this we may neglect $D_{\mathrm{K}} \div D_{\mathrm{Na}}$ (since it is not far from unity) and write as a first approximation

$$\frac{R_{\mathrm{K}}}{R_{\mathrm{Na}}} = \frac{\mathrm{K}'_o - \mathrm{K}'_i}{\mathrm{Na}'_o - \mathrm{Na}'_i}$$

The values of K'_o and Na'_o are kept approximately constant by continually renewing the solution in A. It has been shown elsewhere[5] that the activity of K'_o is proportional to the ionic activity product[17] $(\mathrm{K}_o)(\mathrm{OH}_o)$ in A. Hence we may put $\mathrm{K}'_o = S_{\mathrm{K}_o}\mathrm{K}_o = C_4(\mathrm{K}_o)(\mathrm{OH}_o)$ and it is evident that the value of S_{K_i} will depend on that of OH^-. We may treat K'_i in the same way. All of this applies equally to sodium.

As already stated $\mathrm{K}'_o = S_{\mathrm{K}_o}\mathrm{K}_o$: hence to approximate the value of K'_o a determination of S_{K_o} = (concentration of undissociated KG in the surface of B_o) ÷ (concentration of K^+ in A) is desirable. This cannot be done directly but we may approximate it by determining[18] S_{KG_o} = (concentration of KG in the surface of B_o) ÷ (concentration of K^+ in A). It seems highly probable that the ratio $S_{K_o} \div S_{Na_o}$ is approximately equal to $S_{KG_o} \div S_{NaG_o}$ and since we need comparative values only we may use S_{KG_o} and S_{NaG_o} in place of S_{K_o} and S_{Na_o}.

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[16] This might not have much effect on the value of $\mathrm{K}_i \div \mathrm{Na}_i$ as calculated by the method given on p. 449, since it might influence both of the diffusing substances in similar fashion.

[17] Under the conditions of the experiment the activity of OH in C bears a constant relation to the activity of HCO_3^- and to that of the guaiacol ion G^-.

We may write $\mathrm{K}'_o = C_3(\mathrm{K}_o)(\mathrm{OH}_o)$ and $\mathrm{K}'_i = C_4(\mathrm{K}_i)(\mathrm{OH}_i)$ where the subscripts o and i refer to the outside and inside solutions respectively. But C_3 and C_4 vary with concentration because the non-aqueous phase changes.

The values of S_{KG} and S_{NaG} were determined as follows. An aqueous solution containing equal concentrations of KG and NaG was shaken with a relatively small volume of G. C. mixture on a shaking machine and was then allowed to stand for 24 hours or longer. The G. C. mixture was then shaken with half its volume of 0.08 M HCl which removed practically all of the potassium and sodium. The determinations of potassium and of sodium were made as previously described.[2] The temperature was 21° ± 2°C.

Since the aqueous phase loses more potassium than sodium it must be relatively large to keep its ratio $K_o \div Na_o$ approximately constant or else it must contain more potassium at the start. As an illustration of the latter method we may cite the following: 104 cc. of aqueous solution of 0.222 M KOH + 0.151 M NaOH was shaken with 100 cc. G. C. mixture. After separation there was 98 cc. of the aqueous solution in which the concentrations were 0.107 M potassium and 0.110 M sodium. There was also 106 cc. G. C. mixture in which the concentrations were 0.116 M potassium and 0.053 M sodium. Hence we have $S_{KG_o} = 0.116 \div 0.107 = 1.085$ and $S_{NaG_o} = 0.053 \div 0.110 = 0.482$. Hence $S_{KG_o} \div S_{NaG_o} = 1.085 \div 0.482 = 2.26$.

The situation is easily seen from the following considerations. For the aqueous phase let us put: volume = V, concentration of potassium = C_K, concentration of sodium = C_{Na}, moles of potassium = M_K, moles of sodium = M_{Na}, and designate the corresponding values in the non-aqueous mixture as V', C_K', etc. Assuming that V = 1 liter, V' = 10 liters, and that owing to the nature of the non-aqueous phase the partition coefficient, *i.e.* $C_K' \div C_K$ has a value of 0.4, and that at the start $C_K = C_{Na} = 1$, we have at equilibrium (providing no change in volume occurs and that potassium and sodium act independently)

$$C_K = M_K = 1 - M_K'$$

$$\frac{M_K'}{10} = C_K' = 0.4C_K = 0.4(1 - M_K')$$

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[18] Strictly speaking we do not determine undissociated KG in B_o but total (stoichiometric) concentration of potassium in B_o which amounts to practically the same thing since the concentrations of KOH and $KHCO_3$ in B_o are negligible in comparison with that of KG, and KG is a very weak electrolyte in B (as shown by the unpublished work of physical chemists).

hence

$$M'_K = 0.8$$
$$C_K = M_K = 1.0 - 0.8 = 0.2$$
$$C'_K = 0.8 \div 10 = 0.08$$

Hence $C'_K \div C_K = 0.08 \div 0.2 = 0.4$: this is the value previously assumed for the partition coefficient.

Assuming that the partition coefficient for sodium, *i.e.* $C_{Na}' \div C_{Na} = 0.2$ and proceeding in the same way we obtain for equilibrium $C_{Na} = 0.335$, $C_K \div C_{Na} = 0.2 \div 0.335 = 0.594$; also $C_{Na}' = 0.067$ and $C_K' \div C_{Na}' = 0.08 \div 0.067 = 1.2$. This latter value changes to 1.02 when we put $V = 1$ and $V' = 100$ (instead of $V' = 10$), to 1.71 when we put $V = 1$ and $V' = 1$, and to 1.95 when we put $V = 1$ and $V' = 0.1$.

We see that the greater the relative volume of the aqueous solution the nearer the ratio $C'_K \div C'_{Na}$ in the non-aqueous phase approaches to the ratio of partition coefficients which in this case is $0.4 \div 0.2 = 2$.

A series of values of S_{KG_o} and S_{NaG_o} is shown in Fig. 3. They were determined when both K*G* and Na*G* were present in equal concentrations in the aqueous phase at equilibrium.[19]

Let us now consider conditions at the inner phase boundary. Here the partition coefficients are S_{K_i} and S_{Na_i} (where $S_{K_i} = K'_i \div K_i$ and $S_{Na_i} = Na'_i \div Na_i$). We may use S_{KG_i} = (concentration of K*G* in the surface of B_i) $\div$ (concentration of K^+ in *C*) in place of S_{K_i}.

The determinations are somewhat uncertain because on reaching *C*, K*G* is transformed to $KHCO_3$[5], and we must therefore determine S_{KG_i} by shaking up an aqueous solution of $KHCO_3$ with G. C. mixture so that not only K*G* but also $KHCO_3$ is taken up. We can determine the total amount of potassium taken up but we do not know exactly what percentage of this is K*G*.[20] We can also determine the total carbon taken up but we do not know exactly what per cent of this is HCO_3 and what is CO_2. We find that (concentration of potassium in G. C. mixture) ÷ (concentration of potassium in aqueous phase) varies with concentration and hence we conclude that S_{K_i} varies with concentration. This also applies to S_{Na_i}.

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[19] Adding Na*G* to K*G* affects S_{KG_o} as follows: As stated elsewhere (footnote 5) K'_o is proportional to the ionic product $(K_o)(G_o)$ when activities are taken. Using concentrations (and taking total potassium in *B* as approximately equal to K'_o) we may say that since S_{KG_o} equals (K*G* in *B*) $\div$ (potassium in *A*) the value of K*G* in *B* will be approximately doubled when we double G_o by adding Na*G*: this will double the value of S_{KG_o} since the concentration of potassium in *A* remains constant. This has been tested experimentally and is found to be approximately true.

[20] For example, when 0.63 M $KHCO_3$ at pH 7.5 was shaken up with G. C. mixture the concentration of potassium in the latter was 0.0062 M. The concentration of $CO_2 + HCO_3$ was 0.0025 M. Probably most of this was CO_2: hence if we put $CO_2 = 0.002$ M we have K*G* = 0.0062 − 0.0005 = 0.0057 M. (It depends on the pH value of the aqueous solution.) *Cf.* footnote 5.

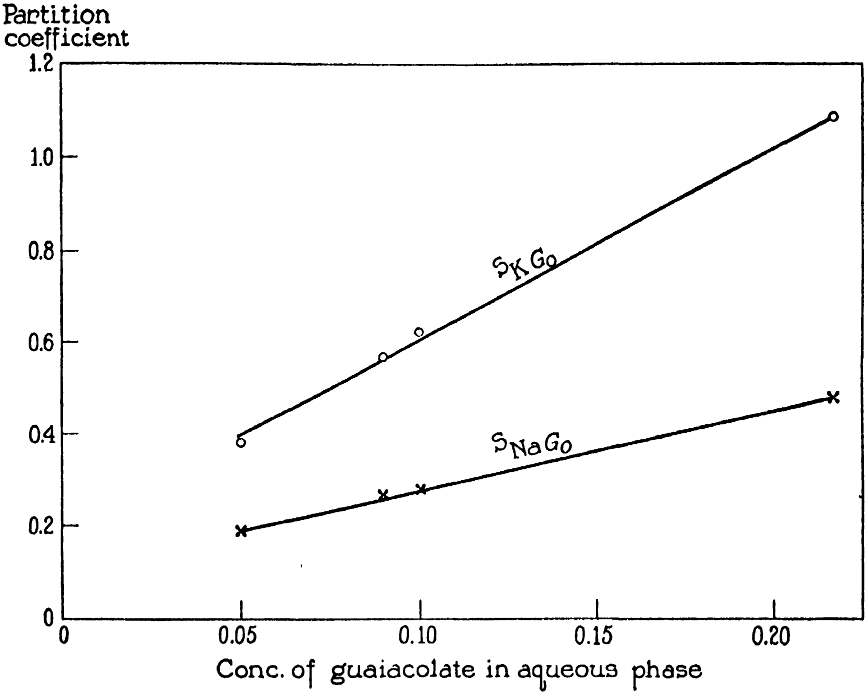

FIG. 3. Values of the partition coefficients S_{KG_0} and S_{NaG_0}. These values were determined in the presence of equal concentrations of potassium and sodium. The total concentration of G^-, or guaiacol ion (taken as equal to $KG + NaG$), is plotted as abscissae. Thus the value for 0.05 means that an aqueous solution of 0.025 M KG + 0.025 M NaG was shaken with a relatively small volume of G. C. mixture and the partition coefficients were calculated from the amounts of KG and NaG found in the G. C. mixture.

Since the ratio $S_{K_i} \div S_{Na_i}$ varies with concentration (independently of pH) and since the concentration of $K_i + Na_i$ may rise during the experiment from zero to 1.2 M there is opportunity for a change in the ratio $K'_i \div Na'_i$ which will affect the relative rates of entrance of potassium and sodium.

The values of S_{K_i} and S_{Na_i} also vary with pH (p. 451). Since the pH value in C may rise[2] from between 5 and 6 to the neighborhood of 7.6 during an experiment the values of S_{K_i} and S_{Na_i} must increase accordingly. Variation in the flow of CO_2 will also affect the pH.

It should be remembered that the diffusion "constants" D_K and D_{Na} as here used are not constant since D_K varies with the concentration of NaG and *vice versa* (p. 447).

Other Factors

It thus appears that penetration depends largely on the diffusion constants and partition coefficients. But it is also affected by a number of other factors, among which are the following:

1. Even when the concentrations in the main body of A are kept constant[2] those at the interface may vary for there is a concentration gradient in the unstirred layer A_B which determines the concentration at the outer surface of B. The more rapid the stirring the thinner this layer and consequently the nearer K_o and Na_o (*i.e.* the concentrations of K^+ and Na^+ at the inner surface of A_B) will approach the concentrations in the main body of A. When the concentrations in A are altered the value of $K'_o \div Na'_o$ may vary because the partition coefficients change. The ratio $K'_o \div Na'_o$ (and therefore of $R_K \div R_{Na}$) will therefore depend in part on the rate of stirring.

This ratio may also be influenced by temperature which alters the viscosity and hence the thickness of the layers (the viscosity is altered by the presence of electrolytes). Temperature may also affect the partition coefficients unequally.

If a model be used with diffusion in B more rapid than in A the ratio $K_o \div Na_o$ may vary because, with potassium moving into B_o more rapidly than sodium, its relative concentration in A_B must become steadily less, unless the rates of stirring and of diffusion be sufficient to renew the supply (see p. 452). As K_o decreases potassium will move more slowly into B.

2. In the stirred layers the forward movement of electrolyte will depend on such factors as rate of stirring, and viscosity.

3. There are concentration gradients in C_B which will depend on the factors already enumerated and which may change the ratio $R_K \div R_{Na}$.

4. Surface forces may play a part; *e.g.*, substances diminishing surface tension will tend to remain in the surface.

5. Reactions may occur at the outer surface and if slow enough may affect the result; *e.g.*, loss of water by the penetrating substance or molecular association in the non-aqueous phase. At the inner surface the reverse processes will occur but here we may have in addition combinations with acids or other substances in *C*.

6. The penetration of electrolytes into *C* is accompanied by that of water. To understand its effect let us consider what happens when water is added suddenly[21] to *C*. Thus if the concentration gradients in *B* be called G'_{K} and G'_{Na} and the ratio be

$$\frac{G'_{\mathrm{K}}}{G'_{\mathrm{Na}}} = \frac{\mathrm{K}'_o - \mathrm{K}'_i}{\mathrm{Na}'_o - \mathrm{Na}'_i} = \frac{0.24 - 0.12}{0.10 - 0.04} = 2$$

and water be added to *C*, doubling its volume, we have

$$\frac{G'_{\mathrm{K}}}{G'_{\mathrm{Na}}} = \frac{0.24 - 0.06}{0.10 - 0.02} = 2.25$$

In this case where the ratio $\mathrm{K}'_o \div \mathrm{K}'_i = 2$ is less than $\mathrm{Na}'_o \div \mathrm{Na}'_i = 2.5$ we observe that the ratio $G'_{\mathrm{K}} \div G'_{\mathrm{Na}}$ rises after the addition of water.

When $\mathrm{K}'_o \div \mathrm{K}'_i$ is greater than $\mathrm{Na}'_o \div \mathrm{Na}'_i$ the ratio falls. Thus if $\mathrm{K}'_o \div \mathrm{K}'_i = 0.24 \div 0.02 = 12$ and $\mathrm{Na}'_o \div \mathrm{Na}'_i = 0.13 \div 0.02 = 6.5$ we have before the addition of water

$$\frac{G'_{\mathrm{K}}}{G'_{\mathrm{Na}}} = \frac{0.24 - 0.02}{0.13 - 0.02} = 2$$

and afterwards

$$\frac{G'_{\mathrm{K}}}{G'_{\mathrm{Na}}} = \frac{0.24 - 0.01}{0.13 - 0.01} = 1.92$$

When $\mathrm{K}_o \div \mathrm{K}_i = \mathrm{Na}_o \div \mathrm{Na}_i$ the addition of water does not change the ratio. Thus if $\mathrm{K}'_o \div \mathrm{K}'_i = 0.24 \div 0.12 = 2$ and $\mathrm{Na}'_o \div \mathrm{Na}'_i = 0.08 \div 0.04 = 2$ we have before the addition of water

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[21] After the sudden addition of water the concentration gradients would no longer be linear.

$$\frac{G'_{K}}{G'_{Na}} = \frac{0.24 - 0.12}{0.08 - 0.04} = 3$$

and afterwards

$$\frac{G'_{K}}{G'_{Na}} = \frac{0.24 - 0.06}{0.08 - 0.02} = 3$$

Evaporation will, of course, produce the opposite effect.

The inward movement of water may have a different temperature coefficient from the movement of the penetrating substances: hence the composition of *C* may depend on temperature.[22]

7. There is an outward movement of substances (from *C* to *A*); *e.g.*, of $KHCO_3$, $NaHCO_3$, CO_2, and H_2CO_3. This may be neglected in the earlier part of the process of penetration but toward the end and in the steady state it may become more important. It is quite possible that the outward movement of $KHCO_3$ may differ from that of $NaHCO_3$ in such fashion as to produce a different ratio of $K_i \div Na_i$ from that which would otherwise occur.

In spite of the fact that penetration is influenced by so many factors the experiments indicate that the ratio of potassium to sodium in *C* depends chiefly on the partition coefficients.

EXPERIMENTS

(*a*) *Diffusion from A to C*

To illustrate this statement we may cite a series of experiments, in which *A* contained equal concentrations of K*G* and Na*G*, *B* contained G. C. mixture, and *C* contained distilled water in which CO_2 was bubbling. The results are shown in Table I (p. 458). It is evident that potassium predominates in *C* in every case as would be expected in view of its higher partition coefficient. We see also that the average of the ratio K ÷ Na in *C* does not differ greatly from $S_{KG_o} \div S_{NaG_o}$. There is considerable variation in the ratios of K ÷ Na: changes during the progress of one experiment are shown in Fig. 4.

Models I, II, and III were used:[2] a steady flow was maintained in *A* thus keeping its composition approximately constant. *A*, *B*, and *C* were stirred. The temperature varied between 20 and 25°C. The determinations of potassium and sodium were made as previously described.[2]

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[22] At the start there may be an outward movement of water since the concentration of electrolytes is greater in *A* than in *B*.

TABLE I

Ratios of Potassium to Sodium in the Artificial Sap in C

Experiment	Time of penetration	Ratio K ÷ Na in *C*		Solution in *A*	Ratio of partition coefficients $S_{KG_0} \div S_{NaG_0}$
	days				
109 *a*	10	2.45	Not in steady state	0.02 M K*G* + 0.02 M Na*G*	2.1
b	8	2.37			
c	11	2.28			
58	9	1.4		0.1 M K*G* + 0.1 M Na*G*	2.3
63	12	1.5			
		Av. = 2.0			
66	33	1.6	In steady state	0.05 M K*G* + 0.05 M Na*G*	2.14
80	4	2.7			
81	4	2.7			
111	50	2.0			
		Av. = 2.3			

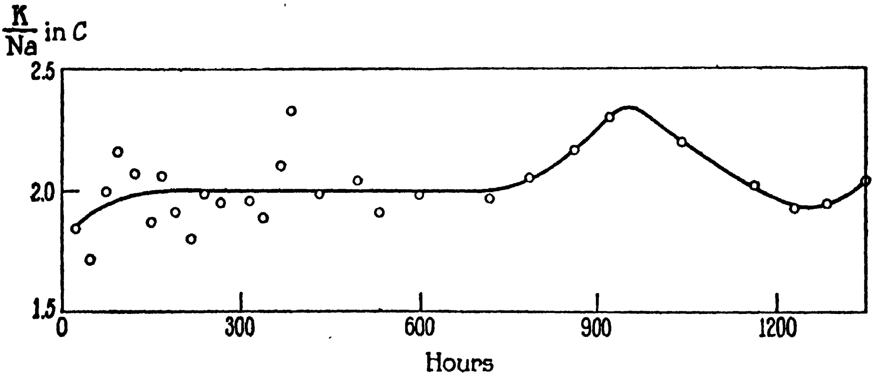

FIG. 4. Ratios of potassium ÷ sodium in *C* in a model in which *A* contained 0.05 M K*G* + 0.05 M Na*G*: *B* contained G. C. mixture, and *C* contained distilled water at the start but had CO_2 bubbling through it throughout the experiment (Experiment 111). The ratio of partition coefficients is $S_{KG_0} \div S_{NaG_0} = 2.14$. The experimental errors are larger during the first part of the experiment when the concentrations in *C* are low.

(*b*) *Diffusion from A to B*

It seemed desirable to examine the process of penetration at each interface separately. To examine the outer interface a model was constructed with *A* and *B* but without *C*. In *A* was placed 0.1 M K*G* + 0.1 M Na*G*, and in *B* was placed G. C. mixture. The results of a typical experiment (No. 83) are shown in Fig. 5.

In this case the concentration gradient of chief importance is that in the layer B_o. Calling the concentration of undissociated K*G* at the outer surface of this layer K'_o and that at the inner limit[23] of this layer (f, Fig. 1) K'_f (with similar designations for sodium) we may write as an approximation

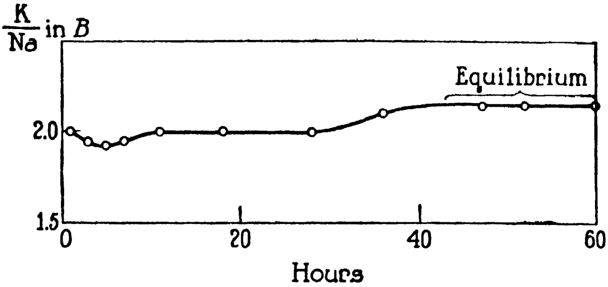

FIG. 5. Ratios of potassium ÷ sodium in *B* in Experiment 83 dealing with the interface between *A* and *B* (*C* was omitted from this model). The ratio of partition coefficients is $S_{KG_o} \div S_{NaG_o} = 2.3$. The experimental errors are larger during the first part of the experiment when the concentrations in *C* are low.

$$\frac{R_K}{R_{Na}} = \frac{K'_o - K'_f}{Na'_o - Na'_f} = \frac{(S_{KG_o}K_o) - K'_f}{(S_{NaG_o}Na_o) - Na'_f}$$

At the start, when K'_f and Na'_f are both equal to zero, we may write as an approximation

$$\frac{R_K}{R_{Na}} = \frac{S_{KG_o}K_o}{S_{NaG_o}Na_o}$$

Since $K_o = Na_o$ we have

$$\frac{R_K}{R_{Na}} = \frac{S_{KG_o}}{S_{NaG_o}}$$

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[23] This is merely a convenient fiction since there is no definite limit at this spot.

We therefore expect at the start a correlation between the partition coefficients and the concentrations of potassium and sodium in *B*. This will also be true as the system approaches equilibrium. Hence it may well be true of the intermediate period and this appears to be the case since the ratio of $S_{\mathrm{K}G_o} \div S_{\mathrm{Na}G_o}$ was found in shaking experiments (p. 452) to be 1.085 ÷ 0.48 = 2.26 which is fairly close to the ratios observed during the progress of the experiment as shown in Fig. 5.

When approximate equilibrium was reached in this experiment the concentration of potassium in *B* was 0.126 M and that of sodium 0.055 M. Hence we have $S_{\mathrm{K}G_o} = 1.26$ and $S_{\mathrm{Na}G_o} = 0.55$: these values are a little higher than those obtained in the shaking experiments[24] (where we found $S_{\mathrm{K}G_o} = 1.085$ and $S_{\mathrm{Na}G_o} = 0.48$. The ratio $S_{\mathrm{K}G_o} \div S_{\mathrm{Na}G_o} =$ 2.29 is also a little higher than the value of 2.26 found in shaking experiments.

Model III was employed.[2] Both *A* and *B* were stirred. A continuous flow in *A* kept the composition constant to within 5 per cent. *B* contained 500 cc. of G. C. mixture. Equilibrium was attained in about 52 hours. The temperature was 20° ± 2°C.

(*c*) *Diffusion from B to C*

In order to examine the processes occurring at the interface between *B* and *C* a model was used in which *B* was brought into equilibrium with *A* (by shaking *B* with a great excess of *A*) before the experiment was started (so that there was relatively little diffusion from *A* to *B* during the experiment): *C* contained only distilled water at the start (no CO_2 was bubbled during the experiment). In this case the chief movement during the early part of the experiment was across the interface between *B* and *C* so that the layer of chief importance is B_i. Calling the concentration of undissociated K*G* at the outer limit[25] (*g*, Fig. 1) of this layer K'_o and that at the other surface K'_i (with similar designations for sodium) we may write as an approximation

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[24] *I.e.* experiments in which a solution containing 0.107 M KOH + 0.110 M NaOH was shaken up with G. C. mixture as described on page 452. As shown in Fig. 3 the ratio $S_{\mathrm{K}G_o} \div S_{\mathrm{Na}G_o}$ is very nearly the same at a concentration of 0.2 M G^- as at a concentration of 0.217 M.

[25] This is, of course, a convenient fiction as there is no sharp limit at this spot.

$$\frac{R_{\mathrm{K}}}{R_{\mathrm{Na}}} = \frac{\mathrm{K}'_o - \mathrm{K}'_i}{\mathrm{Na}'_o - \mathrm{Na}'_i}$$

A typical experiment (Experiment 86) was started with 0.1 M KG + 0.1 M NaG in A, distilled water saturated with guaiacol (no CO_2 bubbling) in C, and with B in equilibrium with A. From the very start B contained 0.1085 M KG and 0.048 M NaG. Since $S_{\mathrm{K}G_o} = 1.085$ and $S_{\mathrm{Na}G_o} = 0.48$ the ratio $S_{\mathrm{K}G_o} \div S_{\mathrm{Na}G_o}$ is 2.26. A simple calculation shows that soon after the start of the experiment $K_i \div \mathrm{Na}_i$ might be in the neighborhood of 1.6 and this agrees fairly well (Fig. 6) with observation (although the validity of this method of calculation may be questioned: see page 449). As $\mathrm{K}_o = \mathrm{Na}_o$ we expect that as equilibrium is approached $\mathrm{K}_i \div \mathrm{Na}_i$ will approach unity and this appears to be the case. The calculation is as follows:

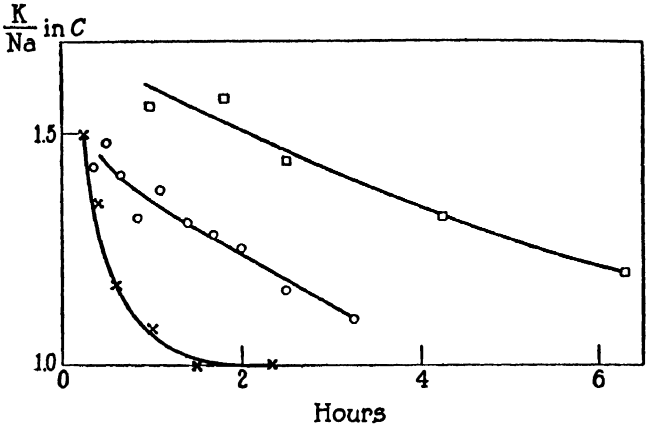

FIG. 6. Ratios of potassium ÷ sodium in C in Experiments 86 a (×), 86 b (○), and 86 c (□), dealing with the interface between B and C. The ratio of partition coefficients is $S_{\mathrm{K}G_o} \div S_{\mathrm{Na}G_o} = 2.3$. The experimental errors are larger during the first part of the experiment when the concentrations in C are low.

Consider a thin layer of B, only a few molecules thick, adjoining the inner interface: we may call this B_i, and the corresponding layer in C on the other side of the interface C_{Bs}. For convenience we put the volume of each of these layers at 1 liter.

We see that B_i, contains 0.1085 mole of KG and we may suppose that when water is brought in contact with it enough KG instantaneously moves into C_{Bs} to bring these two layers into approximate equilibrium. If during this process no more

KG moves into B_i, we may make the following calculation: After approximate equilibrium is reached we have

$$\frac{\mathrm{K}'_i = 0.1085 - \mathrm{K}_i}{\mathrm{K}_i} = S_{\mathrm{K}G_i}$$

If for convenience[26] we put $S_{\mathrm{K}G_i} = S_{\mathrm{K}G_o} = 1.085$ we have

$$\frac{0.1085 - \mathrm{K}_i}{\mathrm{K}_i} = 1.085$$

whence $\mathrm{K}_i = 0.052$ and $\mathrm{K}'_i = 0.0565$. In the same way we have

$$\frac{0.048 - \mathrm{Na}_i}{\mathrm{Na}_i} = 0.48$$

whence $\mathrm{Na}_i = 0.0324$ and $\mathrm{Na}'_i = 0.0156$. Hence $\mathrm{K}_i \div \mathrm{Na}_i = 0.052 \div 0.0324 = 1.6$. We then have for the ratio of gradients

$$\frac{\mathrm{K}'_o - \mathrm{K}'_i}{\mathrm{Na}'_o - \mathrm{Na}'_i} = \frac{0.1085 - 0.0565}{0.048 - 0.0156} = 1.6$$

so that we might expect $R_{\mathrm{K}} \div R_{\mathrm{Na}}$ to be about 1.6 as indeed appears to be the case with the upper curve in Fig. 6 extrapolated to zero time.

Model III was employed.[2] A constant flow in A kept its composition nearly constant: B was brought into equilibrium with A by shaking the two phases together before the experiment started: C contained at the start 75 cc. of distilled water saturated with G. C. mixture and was stirred by a stream of air. A and B were stirred mechanically. The temperature was 20° ± 2°C.

In another sort of experiment[27] (Experiment 66) no CO_2 was bubbled during the first 236 hours and the concentrations in C reached the same level as in A, namely 0.05 M KG + 0.05 M NaG: B was then practically uniform throughout and contained 0.031 M KG + 0.0145 M NaG. The ratio $S_{\mathrm{K}G_o} \div S_{\mathrm{Na}G_o}$ was $0.62 \div 0.29 = 2.14$.

At 236 hours the bubbling of CO_2 began and 4 hours later the ratio K ÷ Na in C was 1.65 (as shown at the ordinate marked 240 in Fig. 7). The ratio rose until the 264th hour after which it slowly fell and then gradually rose again.

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[26] This makes the value of $S_{\mathrm{K}G_i}$ too large but as that of $S_{\mathrm{Na}G_i}$ will be too large by a corresponding amount the error in the ratios $\mathrm{K}'_i \div \mathrm{Na}'_i$ and $\mathrm{K}_i \div \mathrm{Na}_i$ will be small.

[27] Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931–32, **15**, 676, 678 (Experiment 66, Table I, and Fig. 6).

In the steady state[28] C contained 0.73 M $KHCO_3$ + 0.45 M $NaHCO_3$, giving a ratio of 1.62.

Model I was employed. A, B, and C were stirred as usual. A constant flow was maintained in A. The temperature varied from 20 to 25°C. during the course of the experiment.

In concluding the experimental part we may say that in all cases (the diffusion constants being nearly equal) the ratio K ÷ Na in C seems to depend chiefly on the partition coefficients. But other factors are sufficiently influential to produce considerable variation in these ratios.

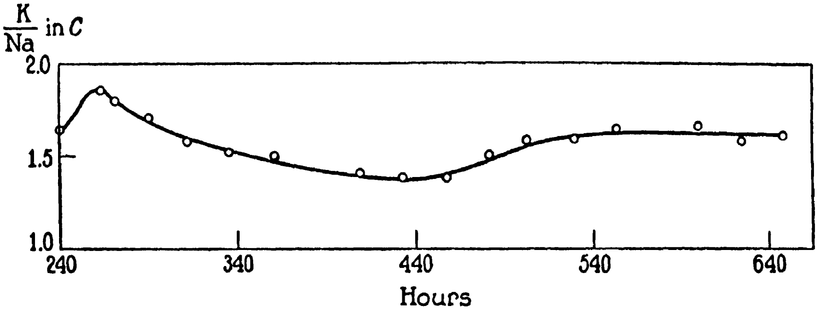

FIG. 7. Ratio of potassium ÷ sodium in C in Experiment 66, dealing with the interface between B and C. During the first part of the experiment no CO_2 was bubbled through C. At 236 hours the ratio in C was unity and B was in equilibrium with A. The bubbling of CO_2 was then commenced and the ratio quickly changed: at 240 hours it was 1.65. The ratio of partition coefficients was $S_{KG_o} \div S_{NaG_o} = 2.2$. In the first part of the curve there are larger experimental errors in determining the concentrations of sodium and potassium (since they are relatively low).

DISCUSSION

It seems probable that many of the variables discussed in this paper are found in living cells such as those of *Nitella* and of *Valonia.*[29]

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[28] At this time A contained 0.043 M KG + 0.007 M $KHCO_3$ + 0.043 M NaG + 0.007 M $NaHCO_3$. The bicarbonate was due to diffusion of HCO_3^- and CO_2 from C into A.

[29] The models would resemble the living cells more closely if we employed KOH and NaOH in A and if KG and NaG, though soluble in the non-aqueous layer, were practically insoluble in water.

In *Valonia* and *Nitella* we apparently have a continuous non-aqueous phase at the inner and outer protoplasmic surfaces.[30] We may suppose that these surface layers correspond to the layers B_o and B_i in the model and that between them is an aqueous phase more or less stirred by protoplasmic movement or by convection currents. The external solution and the sap are well stirred by convection currents. We therefore seem to have opportunity for some of the variables that are found in models.

In these models the chief factors appear to be partition coefficients and diffusion constants. Is this true of living cells in general?

In seeking to answer this question we must remember that the importance of the partition coefficients depends on the speed of diffusion in the non-aqueous layers. When diffusion in these layers is slow enough to control penetration the partition coefficients become important. It would seem that this applies to most living cells since partition coefficients appear to play a highly important rôle. It is for this reason that Overton's theory is so useful, especially as amended by Irwin.

When the partition coefficients of two substances are not very different molecular size becomes important because it determines the diffusion constants. It may also be due in some cases to the fact that penetration is regulated more by the cell wall than by the protoplasm.

In many cases the non-aqueous layers are probably very thin. Hence the diffusion constants in these layers must be very small or the concentration gradients (due to the partition coefficients at the two surfaces of the layer) must be very gentle in order to make diffusion slow enough to have the process of penetration controlled by this layer.

The diffusion constants would, of course, be small if the viscosity were high. It is not necessary to suppose that the layers are solid since protoplasm in contact with water rounds up as though true surface tension existed.[30]

It may be remarked in passing that the idea that protoplasm has a non-aqueous surface has been opposed on the ground that water and salts enter freely. But the model shows that this objection does not hold since water and salts freely pass through the non-aqueous layer.

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[30] Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, **35**, 967.

Let us now consider the question of ionization. In our models K*G* penetrates very rapidly as compared with KCl although both are equally ionized in the external solution. But both are weak electrolytes in the non-aqueous phase and hence penetrate chiefly in molecular form. The difference lies in the fact that K*G* has a much higher partition coefficient than KCl.

There is another consideration which may well be mentioned here, namely, that partition coefficients are important in bioelectric effects. Assuming the latter to be chiefly due to diffusion potentials[31] we may illustrate the situation by means of models. For example, when only K*G* is present the diffusion potential at the outer surface will depend on K'_o and at the inner surface on K'_i. Now when $S_{K_o} = S_{K_i}$ and $K_o \div K_i = 10$ then $K'_o \div K'_i = 10$. But when $S_{K_o} = 1$ and $S_{K_i} = 0.1$, and $K_o \div K_i = 10$ we have $K'_o \div K'_i = 100$ and the diffusion potential increases accordingly. Similar considerations would apply if K*G* were placed at the outer surface and Na*G* at the inner since the concentrations and consequently the potential in the non-aqueous phase would depend on the values of S_{K_o} and S_{Na_i}.

Aside from the question of partition coefficients the outstanding fact brought out by these experiments is the large number of variables concerned. No attempt has been made to treat all of these quantitatively or to set up equations for the time curve of penetration. In a previous paper an empirical equation was given which fits the observations satisfactorily when potassium alone is present and a rigorous treatment has been formulated by L. G. Longsworth[32] by means of which the time curve has been calculated. By means of other equations he has calculated the ratio of sodium to potassium in the steady state. But no attempt has been made as yet to include in this treatment all the variables mentioned in this paper.

The fact that with constant concentrations of potassium and sodium outside there is so much variation in their proportions in the artificial sap in *C* recalls the situation in *Valonia* where there is a considerable variation in the cell sap. It happens that the variation is similar in the two cases for if we divide the highest observed ratio of K ÷ Na by the lowest we get in the model 2.7 ÷ 1.4 = 1.93 and in the case of *Valonia*[1] 5.72 ÷ 2.55 = 2.24.

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[31] Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 715; *Biol. Rev.*, 1931, **6**, 369; *Ergebn. Physiol.*, 1933, **35**, 967.

[32] Longsworth, L. G., *J. Gen. Physiol.*, 1933–34, **17**, 211.

In conclusion it may be appropriate to repeat that when the living cell shows a great difference in the penetration of similar compounds of potassium and of sodium it seems safe to conclude that diffusion coefficients cannot be responsible since the molecular sizes cannot differ greatly. Hence the difference must lie in partition coefficients. This is a very important factor in dealing with living organisms.

SUMMARY

Some of the factors affecting penetration in living cells may be advantageously studied in models in which the organic salts K*G* and Na*G* diffuse from an aqueous solution *A*, through a non-aqueous layer *B* (representing the protoplasmic surface) into an aqueous solution *C* (representing the sap and hence called artificial sap) where they react with CO_2 to form $KHCO_3$ and $NaHCO_3$. Their relative proportions in *C* depend chiefly on the partition coefficients and on the diffusion constants in the non-aqueous layer. But the ratio is also affected by other variables, among which are the following:

1. Temperature, affecting diffusion constants and partition coefficients and altering the thickness of the unstirred layers by changing viscosity.
2. Viscosity (especially in the non-aqueous layers) which depends on temperature and the presence of solutes.
3. Rate of stirring, which affects the thickness of the unstirred layers and the transport of electrolyte in those that are stirred.
4. Shape and surface area of the non-aqueous layer.
5. Surface forces.
6. Reactions occurring at the outer surface such as loss of water by the electrolyte or its molecular association in the non-aqueous phase. The reverse processes will occur at the inner surface and here also combinations with acids or other substances in the "artificial sap" may occur.
7. Outward diffusion from the artificial sap. The outward movement of $KHCO_3$ and $NaHCO_3$ is small compared with the inward movement of K*G* and Na*G* when the concentrations are equal. This

is because the partition coefficients[3] of the bicarbonates are very low as compared with those of Na*G* and K*G*.

Since CO_2 and HCO_3^- diffuse into *A* and combine with K*G* and Na*G* the inward movement of potassium and sodium falls off in proportion as the concentration of K*G* and Na*G* is lessened.

8. Movement of water into the non-aqueous phase and into the artificial sap. This may have a higher temperature coefficient than the penetration of electrolytes.

9. Variation of the partition coefficients with concentration and pH.

Many of these variables may occur in living cells. (It happens that the range of variation in the ratio of potassium to sodium in the models resembles that found in *Valonia*.)

KINETICS OF PENETRATION

VII. Molecular versus Ionic Transport

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In many living cells potassium penetrates more rapidly than sodium. Since the potassium ion has a greater mobility in water than the sodium ion some investigators see in this situation evidence that electrolytes pass chiefly as ions through the protoplasmic surface, in which the order of ionic mobilities is supposed to be that found in water.

If it could be demonstrated that the ionic mobilities correspond in this way[1] it would not show that electrolytes pass chiefly as ions through the protoplasmic surface for such a correspondence might equally well exist if the movement were mostly in molecular form.

This can be seen in certain models in which the protoplasmic surface[2] is represented by a non-aqueous layer B, and electrolytes pass[3] from an aqueous phase A through B into an aqueous phase C (representing the cell sap).

Let us consider a typical experiment in which A contained 0.05 M potassium plus 0.05 M sodium, both combined with guaiacol and *p*-

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[1] Such a correspondence would be expected on the basis of Walden's rule according to which the mobility of an ion is inversely proportional to the viscosity of the medium. Hence the order of mobilities would be the same in all media. This rule seems to work much better for large ions than for small ones (*cf.* Ulich, H., *Tr. Faraday Soc.*, 1927, **23**, 388). On this basis we should expect the ratio of U_K (the mobility of the potassium ion) divided by U_{Na} (the mobility of the sodium ion) to be constant in all media but this may not be the case. In the protoplasmic surface this ratio appears to be very much greater than in water (*cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 715; *Ergebn. Physiol.*, 1933, **35**, 967) if we neglect partition coefficients and phase boundary potentials.

Evidently the result will depend greatly on the degree of solvation and on the formation of complex ions.

[2] Osterhout, W. J. V., *Biol. Rev.*, 1931, **6**, 369.

[3] Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931–32, **15**, 667.

cresol to form organic salts. For convenience the potassium salts will be lumped together and called K*G* (as in previous papers[3,4]). The sodium salts will be called Na*G*.

In *B* was a mixture of 70 per cent guaiacol + 30 per cent *p*-cresol which will be called G.C. mixture. *C* contained at the start distilled water and CO_2 was bubbled through it during the entire experiment.[5]

Under these conditions K*G* penetrated through *B* into *C* where it was transformed to $KHCO_3$ which does not readily pass out; Na*G* acted similarly. As a result potassium and sodium attained much higher concentrations in *C* than in *A*. Eventually a steady state was reached in which the volume of *C* increased while the composition remained approximately constant: the concentration of potassium in *C* in the steady state was about twice that of sodium.

All three phases were stirred but at the phase boundaries there were unstirred layers[3] in which penetration was slow because it depended on diffusion: in the unstirred non-aqueous layers, which may be called B_o and B_i (where the subscripts *o* and *i* refer to the outer and inner surfaces respectively) it was so much slower than elsewhere that these two layers controlled the process of penetration.

The unpublished work of physical chemists shows that K*G* and Na*G* are very weak electrolytes in *B* and we may therefore conclude that these salts move through *B* chiefly in molecular form.

Following the usage of previous papers[3,4,5] we may write as an approximation

$$\frac{R_K}{R_{Na}} = \left(\frac{D_K}{D_{Na}}\right) \frac{K'_o - K'_i}{Na'_o - Na'_i}$$

where R_K is the rate of entrance (in moles) of potassium into *C*, K'_i and K'_o are the concentrations of undissociated K*G* in the outer surface of B_o and the inner surface of B_i respectively, and D_K is the diffusion constant of undissociated K*G* in *B* (a corresponding nomenclature is used for Na*G*).[6]

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[4] Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16,** 529.

[5] This is Experiment 111. See Osterhout, W. J. V., Kamerling, S. E., and Stanley, W. M., *J. Gen. Physiol.*, 1933–34, **17,** 445.

[6] In this equation the dissociation of K*G* and Na*G* in *B* is neglected since it is small and since the values are used only for comparative purposes; *e.g.*, comparison of $K'_o - K'_i$ with $Na'_o - Na'_i$. It should be noted that this formula takes no account of the movement of water.

This may be rewritten

$$\frac{R_{\mathrm{K}}}{R_{\mathrm{Na}}} = \left(\frac{D_{\mathrm{K}}}{D_{\mathrm{Na}}}\right) \frac{\mathrm{K}_o S_{\mathrm{K}_o} - \mathrm{K}_i S_{\mathrm{K}_i}}{\mathrm{Na}_o S_{\mathrm{Na}_o} - \mathrm{Na}_i S_{\mathrm{Na}_i}}$$

where K_o is the concentration of potassium in the inner surface of A, K_i is the corresponding concentration in the outer surface of C: S_{K_o} and S_{K_i} are partition coefficients; *i.e.*, $S_{\mathrm{K}_o} = \mathrm{K}'_o \div \mathrm{K}_o$ and $S_{\mathrm{K}_i} = \mathrm{K}'_i \div \mathrm{K}_i$. A corresponding notation is used for Na*G*.

Since $D_{\mathrm{K}} \div D_{\mathrm{Na}}$ is nearly unity[5] it may be neglected and we may therefore suppose that the relative rate of penetration depends chiefly on the partition coefficients. We should therefore say that potassium predominates over sodium in C because the partition coefficient of K*G* is higher than that of Na*G*.

It is known from the unpublished investigations of physical chemists that the potassium ion has a higher mobility in B than the sodium ion. But it seems unlikely that this plays an important rôle since (*a*) the mobility of K^+ is only slightly greater than that of Na^+ and (*b*) both K*G* and Na*G* are very weak electrolytes in B.[5] Hence in passing through B both K*G* and Na*G* must move chiefly in molecular form and the rôle of ionic mobility is negligible.

In order therefore to explain the more rapid penetration of potassium we must suppose that the chief cause lies in the fact that the partition coefficient of K*G* is larger than that of Na*G* (Table I, p. 477) and in consequence the concentration gradient in B is greater.

We may now ask whether this applies to other substances. In order to test this we have determined the diffusion constants and the rates of penetration of the following pairs of organic alkali salts diffusing together: lithium and potassium; sodium and potassium; sodium and rubidium; and sodium and cesium.

The Northrop diffusion apparatus[7] was used with all the suggested precautions. The temperature was 25° ± 0.1°C.

The alkali G.C. salts were prepared by shaking an aqueous solution of the hydrate with G.C. mixture and then removing the non-aqueous solution by means of a separatory funnel: this was analyzed by the methods previously reported.[3, 5] The solution for the diffusion cell was then prepared by mixing weighed quantities of two G.C. solutions so that the resultant solution contained two G.C. salts in equal concentration, about 0.3 M of each. This mixture was placed inside the cell and 25 cc. of G.C. mixture were then placed outside the cell and diffusion permitted long enough to produce a quantity sufficient for analysis.

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[7] Northrop, J. H., and Anson, M. L., *J. Gen. Physiol.*, 1928–29, **12**, 543.

The amounts diffusing for the pairs of alkalies were determined by shaking the G.C. solution with aqueous 0.1 M HCl and then determining the potassium or sodium content of the HCl extract. In all experiments with sodium present it was determined gravimetrically either by the sodium magnesium uranyl acetate method[8] or by the sodium zinc uranyl acetate method,[9] the latter being used when the quantity of sodium available was less than 8 mg. The other element present was determined by difference.

For the four pairs of alkali G.C. salts, each pair diffusing together in G.C. mixture containing them at equal concentration, the experiments showed the rate of diffusion to be the same within 12 per cent.

In the case of NH_4G the escape of NH_3 into the air was noticeable and in consequence no trustworthy values could be obtained. It seems fairly safe to assume that the diffusion constant of NH_4G does not differ much from those of K*G* and Na*G*.

No great accuracy can be claimed for these results, but they show that the diffusion constants are so similar that the differences between them may be neglected for our present purpose. But it is of interest to note that in general the heavier molecule appeared to diffuse faster which may be due to greater solvation on the part of the lighter molecule.

It is clear that such differences are not great enough to account for the large differences observed in the penetration of these substances in the model (Table I, p. 477). Let us now inquire how far these differences in penetration can be explained by the partition coefficients.

Since it is not possible to determine the partition coefficient S_{K_o}, *i.e.* (the concentration of undissociated K*G* in B_o) ÷ (the concentration of potassium in A), we must content ourselves with determining[5] S_{KG_o}; *i.e.*, (the concentration of potassium in B_o) ÷ (the concentration of potassium in A). There is probably little difference between these values since K*G* is a very weak electrolyte in the G.C. mixture.[5]

The partition coefficients are shown in Fig. 1. It will be noted that at lower concentrations the graphs approximate straight lines. This would be expected for reasons given in former papers.[2,4] At higher concentrations some of the curves bend over and may be approaching a partition coefficient of unity. Determinations of KG, NaG, and LiG showed that the highest concentrations shown in Fig. 1 were not far from the plait points where the two phases fuse and the partition coefficients become unity.

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[8] Caley, E. R., and Foulk, C. W., *J. Am. Chem. Soc.*, 1929, **51**, 1664.

[9] Barber, H. H., and Kolthoff, I. M., *J. Am. Chem. Soc.*, 1928, **50**, 1625.

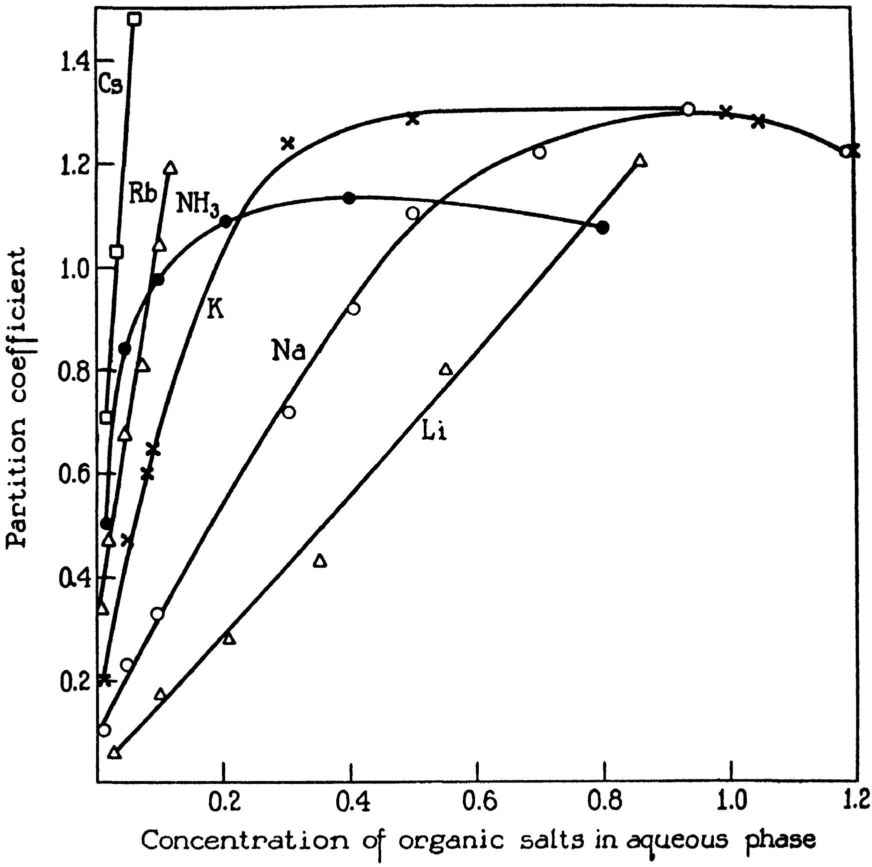

FIG. 1. Graphs showing values of partition coefficient $\frac{MG \text{ in non-aqueous phase}}{G^- \text{ in aqueous phase}}$, where M is an alkali cation: G^- in the aqueous phase is regarded as equal to M in the aqueous phase. Up to about 0.1 M the graphs are approximately straight lines. This would be expected on theoretical grounds. The curve is drawn free-hand to give an approximate fit to the observed values.

Let us now consider the partition coefficients observed when a pair of salts (such as KG and NaG) are present simultaneously. In this case it is not sufficient to take the values represented in Fig. 1 which shows, for example, that at 0.02 M we have $S_{KG_o} \div S_{NaG_o} = 0.25 \div 0.125 = 2$. The value of S_{KG_o} shown in the figure was obtained from a solution containing 0.02 M K*G* and that of S_{NaG_o} from a solution containing 0.02 M Na*G*. But we must now use[10] the values of S_{KG_o} and S_{NaG_o} in a solution containing 0.02 M K*G* + 0.02 M Na*G*. We then get $S_{KG_o} \div S_{NaG_o} = 0.35 \div 1.65 = 2.12$. The values of S_{KG_o} and S_{NaG_o} are higher than those shown in Fig. 1 because the concentration of guaiacol ion (G_o) and of (OH_o) is higher, which, as shown in previous papers, increases the value of the partition coefficient. This follows from the fact that K'_o is proportional to $(K_o)(G_o)$ and to $(K_o)(OH_o)$ when we take activities.[5] (We can calculate on this basis with sufficient accuracy for our purpose by using concentrations: this is demonstrated by making a series of determinations.)

The following experiments were made to ascertain the relation between partition coefficients and rates of entrance. The analytical procedure was similar to that for the diffusion experiments (p. 472). In all cases *C* contained distilled water at the start and CO_2 was bubbled continuously throughout the experiment.

(*a*) *Potassium and Lithium.*—The outer aqueous phase *A* contained 0.02 M K*G* + 0.02 M Li*G*. After 8 days the ratio K ÷ Li in *C* was 0.178 M ÷ 0.058 M = 3.1 (Experiment 108 *a*). A repetition gave in 8 days 0.250 M ÷ 0.088 M = 2.84 (Experiment 108 *b*). A similar experiment gave 0.0814 M ÷ 0.0261 M = 3.12 after 7 days (Experiment 108 *c*): the liquid in *C* was then removed and fresh distilled water (with CO_2 bubbling through it) was substituted. After 9 days the ratio in *C* was 0.0852 M ÷ 0.0229 M = 3.72 (Experiment 108 *d*). The average ratio was 3.09. Since the highest concentration reached in *C* was K*G* = 0.250 and Li*G* = 0.088 the system was far from the steady state.

A determination of the partition coefficients with 0.02 M K*G* + 0.02 M Li*G* in the aqueous phase gave $S_{KG_o} \div S_{LiG_o} = 0.414 \div 0.08 = 5.2$.

In the experiments here described Model II[3] was used for all the experiments in which Solution *A* contained the two alkalies at 0.02 M each. A constant flow was maintained in *A*: *B* contained 275 cc. of G.C. mixture and *C* 20 cc. of distilled water at the start, and CO_2 was bubbled continuously throughout the experiment. The temperature varied between 20 and 25°C. The other experiments listed in Table I, with the solution in *A* containing potassium and sodium at 0.05 M each and 0.1 M each, have been previously described.[3, 5]

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[10] These determinations were made as described on p. 472. The resulting solution of chlorides was analyzed for potassium and sodium as already described (*cf.* footnote 3).

(*b*) *Sodium and Ammonium.*—The outer phase A^{11} contained 0.02 M Na*G* + 0.02 M NH_4G. After 13 days the ratio in *C* was $NH_4 \div Na = 0.1925$ M $\div$ 0.0375 M = 5.1 (Experiment 106 *a*). A repetition gave after 8 days the ratio 0.1572 M $\div$ 0.0238 M = 6.6 (Experiment 106 *b*): the solution in *C* was then replaced by distilled water in which CO_2 was bubbling and after 9 days we found 0.193 $\div$ 0.0371 = 5.2 (Experiment 106 *c*). Fresh distilled water + CO_2 was placed in *C* and after 8 days we found 0.192 M $\div$ 0.037 M = 5.2 (Experiment 106 *d*). Fresh distilled water plus CO_2 was again placed in *C* and after 7 days we found 0.1731 M $\div$ 0.0289 M = 6.0. The average ratio was 5.6.

A determination of the partition coefficient with 0.02 M Na*G* + 0.02 M NH_4G in the aqueous phase gave $S_{NH_4G_o} \div S_{NaG_o} = 0.885 = 0.15 = 5.9$.

(*c*) *Rubidium and Sodium.*—The outer phase[11] *A* contained 0.02 M Rb*G* + 0.02 M Na*G*. After 4 days the ratio in *C* was $Rb \div Na = 0.0590$ M $\div$ 0.0252 M = 2.34 (Experiment 113 *a*). Fresh distilled water plus CO_2 was then placed in the *C* compartment. After 7 days the ratio in *C* was $Rb \div Na = 0.1094$ M $\div$ 0.0372 M = 2.94 (Experiment 113 *b*).

A determination of the partition coefficient with 0.02 M Rb*G* $\div$ 0.02 M Na*G* in the aqueous phase gave $S_{RbG_o} \div S_{NaG_o} = 0.63 \div 0.181 = 3.5$.

(*d*) *Cesium and Sodium.*—The outer phase[11] *A* contained 0.02 M Cs*G* + 0.02 M Na*G*. After 4 days the ratio in *C* was $Cs \div Na = 0.0548$ M $\div$ 0.0115 M = 4.8 (Experiment 114 *a*). The solution in *C* was replaced by 20.0 cc. distilled water in which CO_2 was bubbling and after 9 days we found $Cs \div Na = 0.0958$ M $\div$ 0.0228 M = 4.2.

A determination of the partition coefficients with 0.02 M Cs*G* + 0.02 M Na*G* in the aqueous phase gave $S_{CsG_o} \div S_{NaG_o} = 1.29 \div 0.135 = 9.6$.

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[11] The conditions were the same as in Experiment 108.

(*e*) ***Potassium and Sodium.***—The outer phase *A* contained 0.02 M K*G* + 0.02 M Na*G*. After 10 days the ratio K ÷ Na in *C* was 0.103 M ÷ 0.042 M = 2.45 (Experiment 109 *a*). A repetition gave in 8 days 0.097 M ÷ 0.041 M = 2.37 (Experiment 109 *b*). A third experiment gave 0.107 M ÷ 0.049 M = 2.28 after 11 days (Experiment 109 *c*). The average ratio was 2.37.

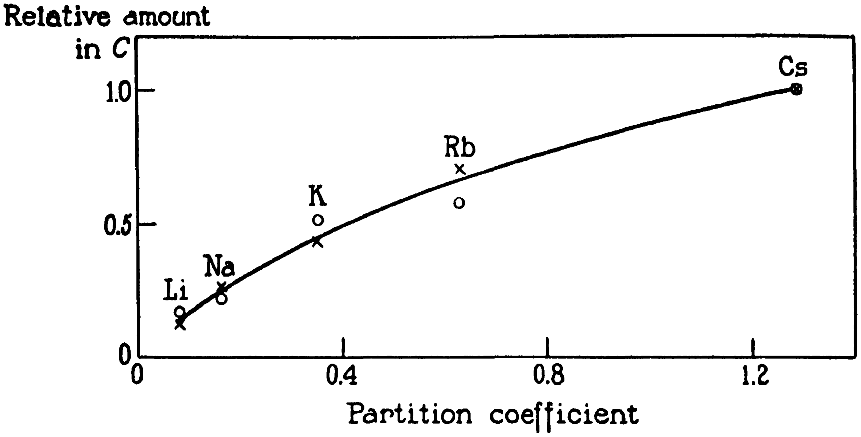

FIG. 2. Graph showing that the salt which predominates in *C* is the one with the higher partition coefficient, which is defined as $\frac{MG \text{ in non-aqueous phase}}{G^- \text{ in aqueous phase}}$ where *M* is an alkali cation: G^- in the aqueous phase is regarded as equal to *M* in the aqueous phase. The partition coefficients are plotted as abscissae (see Table II, p. 478). The relative amount in *C* is plotted as ordinates, that of cesium being taken as unity (see Table II). The curve is drawn free-hand to give an approximate fit to the observed values. Observed values, o; calculated values, ×.

The partition coefficients for 0.02 M K*G* + 0.02 M Na*G* interpolated from determinations at several concentrations for these salts (when present in equal concentration) are $S_{KG_o} \div S_{NaG_o} = 0.35 \div 0.165 = 2.1$.

The results are summarized in Tables I and II. Fig. 2 shows[12] that as the partition coefficient increases the relative amount found in *C* also increases but the curve flattens out rather rapidly. This would

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[12] For reasons which will be discussed presently ammonium is omitted from this figure.

TABLE I

Summary of Penetration Experiments with Alkali Salts

Experiment	Time	Ratio found in C	Solution in *A*	Ratio of partition coefficients
		K ÷ Na		
	days			
58	9	1.4	0.1 M K*G* + 0.1 M Na*G*	$S_{KG_o} \div S_{NaG_o} = 2.3$
63	12	1.5		
		Av. = 1.45		
66	33 Steady state	1.6	0.05 M K*G* + 0.05 M Na*G*	$S_{KG_o} \div S_{NaG_o} = 2.14$
80	4	2.7		
81	4	2.7		
111	50	2.0		
		Av. = 2.25		
109 *a*	10	2.45	0.02 M K*G* + 0.02 M Na*G*	$S_{KG_o} \div S_{NaG_o} = 2.1$
b	8	2.37		
c	11	2.28		
		Av. = 2.37		
		K ÷ Li		
108 *a*	8	3.1	0.02 M K*G* + 0.02 M Li*G*	$S_{KG_o} \div S_{LiG_o} = 5.2$
b	8	2.84		
c	7	3.12		
d	9	3.72		
		Av. = 3.09		
		NH_4 ÷ Na		
106 *a*	13	5.1	0.02 M NH_4G + 0.02 M Na*G*	$S_{NH_4G_o} \div S_{NaG_o} = 5.9$
b	8	6.6		
c	9	5.2		
d	8	5.2		
e	7	6.0		
		Av. = 5.62		
		Rb ÷ Na		
113 *a*	4	2.34	0.02 M Rb*G* + 0.02 M Na*G*	$S_{RbG_o} \div S_{NaG_o} = 3.5$
b	6	2.94		
		Av. = 2.64		
		Cs ÷ Na		
114 *a*	4	4.8	0.02 M Cs*G* + 0.02 M Na*G*	$S_{CsG_o} \div S_{NaG_o} = 9.6$
b	9	4.2		
		Av. = 4.50		

be expected on the basis of the calculation given in the previous paper[13] where a method is presented for calculating $K_i \div Na_i$ after the first increment of time. Employing this we obtain the values given in Column 3 of Table II.

The agreement with the observed values is surprisingly good when it is remembered that (in addition to the difficulties mentioned in the previous paper[13]) the determinations were not made at the beginning but at later stages and even in the steady state.

TABLE II

Relative Amounts Found in C, That of Cesium being Taken As Unity (in Determining These the Averages for 0.02 M in Table I Were Employed)

	Relative amount in *C*		Partition coefficient
	Observed	Calculated	
Cs	1.00	1.00	1.29
Rb	0.58	0.71	0.63
K	0.52	0.44	0.35*
Na	0.22	0.26	0.165*
Li†	0.17	0.13	0.08
NH_4	1.25		0.88

* The partition coefficient of Na*G* depends on the substance with which it is paired: thus with K*G* it is 0.165, with Rb*G* 0.181, with Cs*G* 0.135, and with NH_4G 0.152: the partition coefficient of K*G* is 0.414 with Li*G*, and 0.35 with Na*G*. In constructing Fig. 2 it was necessary to take single values and those given in Table II were selected as the best established since more determinations were made. The differences in the values for S_{KG_0} and S_{NaG_0} depending on the alkali accompanying it may be due to a number of factors, including variations in temperature and in the composition of the G.C. mixture.

† The relative amount (both calculated and observed) of lithium was related to that of cesium by relating cesium to sodium, sodium to potassium, and, finally, potassium to lithium.

Furthermore in these experiments CO_2 was bubbling in *C* and water was entering: neither of these factors was taken into account in the calculation.

We see that both calculation and observation indicate that the rate of penetration is not directly proportional to the partition coefficient, but increases somewhat less rapidly. This may also be the case with living cells.

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[13] Osterhout, W. J. V., Kamerling, S. E., and Stanley, W. M., *J. Gen. Physiol.*, 1933–34, **17**, 449.

DISCUSSION

The order of partition coefficients is

$$\text{Cs} > \text{NH}_4 > \text{Rb} > \text{K} > \text{Na} > \text{Li}.$$

That of rates of penetration is

$$\text{NH}_4 > \text{Cs} > \text{Rb} > \text{K} > \text{Na} > \text{Li}.$$

The fact that ammonium appears to penetrate rapidly is quite in accord with biological experience for we find that in general its penetration into living cells is relatively rapid. The reason usually given is that in addition to penetrating as ammonium (either free or combined) it can penetrate as NH_3. This would also apply to the models. If, for example, *B* contained undissociated NH_4G, NH_4OH, and NH_3, the last would have the highest diffusion constant on account of its small molecular weight. In addition to this it is quite possible that the partition coefficient is really higher than the analysis shows owing to loss of NH_3 during the determination. We may therefore regard ammonium as not strictly comparable to the other salts.

Considering only cesium, rubidium, potassium, sodium, and lithium we see that the order of penetration corresponds to that of their partition coefficients and to that of the ionic mobilities in water. Hence it would seem that similar causes determine the order of ionic mobilities in water and the order of partition coefficients. This will be discussed in forthcoming papers by physical chemists: since their work indicates that these salts are weak electrolytes in *B* we may conclude that ionic transport in *B* plays a subordinate rôle and that the partition coefficients are chiefly responsible for the order of penetration.

The predominating effect of the partition coefficient is also shown in experiments with KCl. Here the diffusion coefficient is presumably greater than for K*G* but in spite of this the rate of penetration is extremely small, corresponding to the very small partition coefficient.

SUMMARY

In some living cells the order of penetration of certain cations corresponds to that of their mobilities in water. This has led to the

idea that electrolytes pass chiefly as ions through the protoplasmic surface in which the order of ionic mobilities is supposed to correspond to that found in water.

If this correspondence could be demonstrated it would not prove that electrolytes pass chiefly as ions through the protoplasmic surface for such a correspondence could exist if the movement were mostly in molecular form.

This is clearly shown in the models here described. In these the protoplasmic surface is represented by a non-aqueous layer interposed between two aqueous phases, one representing the external solution, the other the cell sap.

The order of penetration through the non-aqueous layer is

$$\mathrm{Cs} > \mathrm{Rb} > \mathrm{K} > \mathrm{Na} > \mathrm{Li}.$$

This will be recognized as the order of ionic mobilities in water. Nevertheless the movement is mostly in molecular form in the non-aqueous layer (which is used in the model to represent the protoplasmic surface) since the salts are very weak electrolytes in this layer.

The chief reason for this order of penetration lies in the fact that the partition coefficients exhibit the same order, that of cesium being greatest and that of lithium smallest.

The partition coefficients largely control the rate of entrance since they determine the concentration gradient in the non-aqueous layer which in turn controls the process of penetration. The relative molecular mobilities (diffusion constants) in the non-aqueous layer do not differ greatly. The ionic mobilities are not known (except for K^+ and Na^+) but they are of negligible importance, since the movement in the non-aqueous layer is largely in molecular form. They may follow the same order as in water, in accordance with Walden's rule.

Ammonium appears to enter faster than its partition coefficient would lead us to expect, which may be due to rapid penetration of NH_3. This recalls the apparent rapid penetration of ammonium in living cells which has also been explained as due to the rapid penetration of NH_3.

Both observation and calculation indicate that the rate of penetration is not directly proportional to the partition coefficient but increases somewhat less rapidly.

Many of these considerations doubtless apply to living cells.

BLAIR'S "CONDENSER THEORY" OF NERVE EXCITATION

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(Accepted for publication, November 8, 1933)

In four recent papers, Blair (1931–32; 1932–33) has developed a mathematical concept which he believes to underlie the excitation process in nerve and muscle, and he urges that a deeper insight will be gained into this process if observations are expressed in terms of the equations which he has derived. Since on the one hand many experimenters do not easily digest the somewhat rich diet of mathematics provided in these papers, and since on the other the author appears to have overlooked certain defects in his theory, it is proposed in this note to point out in non-mathematical terms some qualitative advantages and quantitative objections to Blair's treatment.

Prolonged Currents

When a constant current is applied to a tissue, the excitatory state first quickly rises and then (if the threshold is not reached) slowly declines, though not to zero. It is thus possible by increasing a current in small steps to apply without response a current much in excess of the rheobase. If the steps are infinitesimal the case becomes that of slowly rising currents. This property appears to be one of the fundamental facts to be answered in any comprehensive theory. It enters into all discussions of Nernst's hypothesis under his term "accommodation" and has proved the stumbling block in many treatments. In particular one of the limitations of the "condenser theory" is that it will not contemplate this property of nerve and muscle.

If a tissue is represented as a shunted condenser (Fig. 1) it is apparent that when a certain voltage is applied and retained constant, the charge on the condenser will rise to a value characteristic of that voltage, and become quite independent of whether the voltage initially rose suddenly or exceedingly gradually. And this which is intuitively clear from electrical considerations equally follows from Blair's equations which are the exact mathematical equivalent. It is consequently obvious that the condenser theory is quite inapplicable to cases of prolonged currents, because the tissue "accommodates" and the condenser does not.

Blair, however, attempts to apply his equations to the cases of slowly rising currents, but shelves the only significant aspect of the question—the minimum gradient—by suggesting that this is due to some phenomenon different from the local excitatory process.

With regard to the opening excitation, which originates at the anode, it has been usual to regard this as some sort of rebound after the process of "accommodation." If this is the case the consideration were well postponed until some clearer views are available concerning the minimum gradient—which is probably closely related. Blair, however, supposes that the opening excitation is due to a sudden shift in the sensitivity of the tissue so that a certain magnitude of the excitatory process, inadequate to excite while the current is flowing, suddenly becomes adequate owing to the threshold falling faster than the rate of decay of the excitatory process.

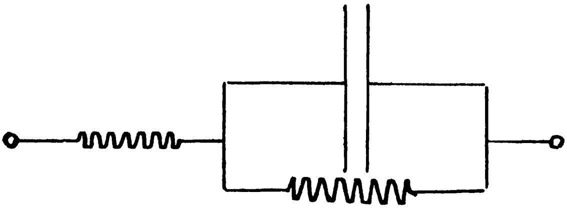

FIG. 1

Since this requires that the opening excitation should occur at that electrode where excitability was enhanced during the current flow, namely the cathode, this concept is quite untenable.

These considerations emphasise that whatever may be the advantages of the condenser theory for brief currents, the theory unmodified has no place at all in relation to prolonged currents.

It is very easy to make a suitable modification, for instance by the assumption that there is polarisable connective tissue in series with the tissue. This accounts qualitatively for all the chief results with prolonged currents—the minimum gradient, the opening excitation, etc.,—and if this polarisation is represented as a leaky condenser it is easy to derive the appropriate equations. But what is required is the physical investigation of what polarisation does in fact occur, not what equations result from supposing that such a polarisation behaves like a parallel plate condenser.

Brief Currents

The fact that the condenser theory is inapplicable to conditions involving "accommodation" does not in any way invalidate it as a theory applicable only to brief currents, for in these cases "accommodation" whatever it is, may be regarded as insufficiently developed to be significant.

I wish to suggest that in this domain the condenser theory is a useful qualitative guide, and a valuable basis upon which to build a more accurate concept of the excitatory process. But both in its simplest form and in the modification by Blair it is quantitatively inadequate.

Blair does not explicitly assume that the tissue is to be regarded as a shunted condenser (Fig. 1), but he postulates that it obey the mathematical law which governs the flow of electricity in such a condenser system, and hence all the results which he derives are identical with those on the condenser theory.

Actually he claims that a nerve cannot be regarded as a condenser since otherwise an alteration in series resistance would change the time relations. This argument falls to the ground if the condenser is assumed shunted by a resistance small compared with the series resistance. The recent papers of Umrath (1930) and Eichler (1931) deal with this matter experimentally and in detail.

Turning now to Blair's form of the condenser theory, we note that he first makes the assumption that the condenser must attain a certain fixed charge h in order that excitation may occur. This is a very reasonable physical concept (and by no means a new one) but it has the disadvantage that it does not accurately fit the facts. To remedy this the assumption is now modified, and the charge must attain not h but $h + \alpha V$, where V is the applied voltage at the moment of excitation, and α is a constant. There is no physical justification for this assumption nor can I find a physical meaning to it. It appears, moreover, to be contrary to facts.

In many preparations Blair claims that α is positive; let us consider what happens in this case according to Blair's hypothesis. If the tissue is excited by a constant current of voltage V, the threshold during the whole period of current flow will be $h + \alpha V$, but the instant after the current ceases the threshold will have fallen to h. Now the excitatory process takes a finite time to decay, hence it will not have diminished appreciably between the instant before and the instant after breaking the stimulating circuit. Consequently, if the excitatory process had attained the value h before breaking, it would have attained this value the instant after breaking and consequently would cause excitation. We must therefore conclude that where α is positive the threshold required will not be $h + \alpha V$ but still h.

But not only does the hypothesis which we are considering bring the results no nearer the facts but it also involves two conclusions of a very startling kind. For if the rheobase current be stopped after flowing for a duration just greater than the utilisation period it need only be such a strength as will cause the excitatory process to attain h. If, however, the rheobase be continued indefinitely so that excitation is observed to occur while the current is still flowing, then the excitatory process must attain the value $h + \alpha V_R$ where V_R is the rheobase in this case. It is obvious that according to this a *higher* threshold is required for a current which continues than for one which is stopped, which is very contrary to experience.

Again it appears that at the instant of starting the stimulus the threshold will rise from h to $h + \alpha V$. If V is negative (*i.e.* the point we are considering is anode) the threshold will not rise but fall when the current starts, and if V is made large enough the threshold will fall below zero; *i.e.*, the tissue will be excited by the resting value of the excitation process. This result is surprising; it signifies that with strong currents, excitation should arise at the anode, the current need not flow for any finite duration, nor is the threshold reduced by increased duration of flow.

Nothing would be served by dwelling further upon Blair's modification of the condenser theory, for we have seen that it is unrelated to any likely physical mechanism, that it does not in fact fit observations any better than the unmodified theory and that it involves consequences of a totally inadmissible kind.

CONCLUSIONS

With regard to the advantages of the condenser theory, they have been urged by so many authors from time to time in connection with so many different experimental investigations, that it is impossible to treat the matter in this place. Suffice it to state that although it rarely happens that the theory fits accurately the observations, yet over a very large range there is a good qualitative correspondence, and this is illustrated in the papers of Blair. In particular in the calculations relating to strength-duration curves and voltage-capacity curves, the latter relation was determined from the former without any arbitrary constants at all, and the correspondence is sufficiently striking.

The following conclusions therefore seem permissible.

The condenser theory in its simple form though quite misleading when applied to cases of prolonged currents is a useful qualitative guide where brief currents are concerned.

The semiquantitative correspondence which subsists between theory and observation in a very wide field suggests that something equivalent to the condenser mechanism may underlie the phenomena of excitation as commonly measured. The particular modification of this theory put forward by Blair, however, is not physically plausible and leads to inadmissible conclusions.

SUMMARY

Blair's recent theory of excitation is analysed with the following conclusions:

1. The theory is inapplicable to currents of long duration; *i.e.*, slowly increasing currents and the opening excitation.

2. The theory is a modification of the condenser theory of excitation but the modification is to be rejected on three grounds:

(*a*) The modification has no obvious physical significance.

(*b*) It does not in fact remedy the divergence between calculation and observation.

(*c*) It leads to certain conclusions of a surprising kind which are contrary to observed fact.

3. The qualitative value of the condenser theory is demonstrated by the fairly close agreement between calculation and observation over a considerable field of enquiry.

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CORRECTIONS

In Vol. **17**, No. 3, January 20, 1934, page 328, formula (2) should have included in the numerator a series of major parentheses; *i.e.*, for

$$\text{percentage of bound water} = \frac{\Delta a - \frac{1000}{892}\Delta + Km}{\Delta a - \frac{1000}{892}\Delta} \times 89.2$$

read

$$\text{percentage of bound water} = \frac{\Delta a - \left(\frac{1000}{892}\Delta + Km\right)}{\Delta a - \frac{1000}{892}\Delta} \times 89.2$$

In Vol. **17**, No. 3, January 20, 1934, in the equation in the center of page 368, a decimal point has been omitted. For $\eta = \frac{1 + 05\,\phi}{(1 - \phi)^4}$, read $\eta = \frac{1 + 0.5\,\phi}{(1 - \phi)^4}$.

THE TIME CURVE OF FACET DETERMINATION IN AN ULTRABAR STOCK OF DROSOPHILA MELANOGASTER

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(Accepted for publication, August 25, 1933)

In the bar series of *Drosophila* flies developing from larvae which have been transferred from one temperature to another before the onset of the facet determination period have the average facet number characteristic for flies which have spent the whole period of their development at the temperature to which the transfer has been made. If the transfer is made after the end of the determination period, then the average facet number is characteristic for flies which have spent the whole period of their development at the temperature from which the transfer has been made. If the transfer has been made during the effective period some intermediate number of facets results.

Such relations are illustrated by the data and figures published by Luce (1931) for infrabar flies. From his Table 2 and Fig. 3 it can be seen that infrabar females which have been transferred as larvae to 17° at any time before the 64th hour of their development at 27° have approximately 131 facets, which is the average number of facets that infrabar females possess if the whole period of their development is passed at 17°. When the larvae are transferred to 17° at any time after the 89th hour of their development at 27°, the resulting flies have approximately 272 facets which is the average number of facets that infrabar females possess when the whole period of their development is passed at 27°. Flies developing from larvae transferred to 17° at some time after the 64th and before the 89th hour of their development has been passed at 27° have a facet number intermediate between 131 and 272 facets. For example, the flies developing from larvae transferred to 17° at the end of the 80th hour of their development at 27° have on the average 222 facets. The curve of the directly observed data connecting the two lines for the facet levels of the upper and lower temperatures (Luce, Fig. 3) is thus a compound curve. Each point on the curve is the sum of a certain number of facets determined at the beginning of the effective period at 27° plus a certain number which are produced presumably in the latter part of the period at 17°. If we knew for different points on the compound curve the two component values, then these data would allow one to reach in this indirect manner the time course of facet determination.

When the length of the effective period is known for the two temperatures, along with the average number of facets produced at these temperatures, and also the number of facets produced for different times of transfer during the effective period, then all the available data are in hand for attempting the dissection of the compound curve into its two components. The simplest possible assumption to make for proceeding with the analysis of the data is that if a certain percentage (x) of the effective period is completed at the temperature from which the transfer is made, then the remainder ($100-x$) is passed at the temperature to which the transfer is made and at the rate characteristic for the temperature concerned. It will be shown below that the data may be quite satisfactorily analyzed on the basis of this assumption. One may, however, doubt the correctness of the assumption, but whatever error is involved does not seem to be larger than the experimental error. A further assumption which needs to be made for undertaking the analysis is in regard to the form of the function descriptive of the time course of facet determination. The simplest assumption in this regard is that the time curve is linear. This assumption does not lead to satisfactory results. Likewise the assumption that the increase in facets is a simple exponential function of time is inconsistent with the data. The assumption on the basis of which the results to be discussed were obtained was that of a symmetrical sigmoid curve. Since consistent results are obtained a presumption is created that the assumptions made are to be accepted as being in agreement with the facts but one may not conclude that the resulting equations necessarily give the form of the true function. To draw such a conclusion it would need to be shown further that the form of function which gives consistent results is the only one that could possibly apply.

The problem may be formulated analytically in the following terms: The number of facets (y) in flies resulting from the transfer of larvae in the effective period is a function of the time (t) spent in the effective period at the temperature from which the transfer is made, that is,

$$y = f(t)$$

This function gives the compound curve. Of the number of facets (y) at any value of t, some are produced at the temperature from which the transfer is made and the remainder at the temperature to which the transfer is made. Any particular value of the function is consequently the sum of two definite integrals.

$$y = \int_0^x \frac{dy}{dt}_{27^\circ} + \int_x^{100} \frac{dy}{dt}_{17^\circ}$$

The first term to the right in the equation gives the number of facets determined during a certain percentage of the effective period at the temperature from which the transfer was made, indicated as 27°, while the second term gives the number of facets determined during the remainder of the effective period at the temperature to which the transfer was made, indicated as 17°. The terms under the integral signs are in each case the first derivative with respect to time of the function describing the time course of facet determination at each temperature. And since we make the assumption of a symmetrical sigmoid curve then for each temperature

$$\frac{dy}{dt} = ky\ (A - y)$$

in which A is the upper asymptote, given by the average number of facets produced at each temperature when the whole period is passed at one temperature, and k is the velocity constant to be estimated from the dissected data. When integrated this equation in straight line form becomes

$$\log \frac{y}{A - y} = K\ (t - t_1)$$

in which $K = kA$ and t_1 is the time when the period is half completed.

The determination of the compound curve, $y = f(t)$ for the data obtained by the method of transferring larvae from one temperature to another would in some cases be obviously complicated and its analytic dissection into the two components would be by no means a simple task from the standpoint of the application of the necessary mathematics. (Indeed I am not aware that mathematics has received any extensive application in this direction.) A less elegant method, but one which need not lead necessarily to inferior results, is to make a simple arithmetic dissection of the data by trial and error and then to determine K in the above equation from the resulting data. This was the method used in reaching the results given below.

The purpose of this paper is to present the results of the application of this method to the data on ultrabar males and females contributed by E. C. Driver (1931). The necessary data were made available to the writer by the courtesy of The Wistar Institute where they were placed on file by Dr. Driver.

The object of the analysis is to determine the course of the reaction, or in other words, the constants in the above equation. A is given directly by the average number of facets possessed by males or females when the whole period of development is passed at one temperature and these values are given in Table II of the filed data. From Tables IX to XIII inclusive of the filed data the limits of the effective period may be determined and so t_1 of the above equation becomes known. The effective periods estimated from the data are a little longer on the average than the results from the same data given by Driver. The method used by Driver (1931, p. 10) was to take "the point at which the plotted line of facet count by time shows the first definite departure from the count of flies at one or other of the two constant temperatures, or, if this line is intersected, the point of intersection." The same general method was followed by the writer except that a double logarithmic plot was used. An advantage of this method is that it tends to make somewhat sharper the points which give the limits of the effective period.

We are especially interested in the value of k, the velocity constant, which may readily be known from the relation $K = kA$. Values for the determination of K can be dissected from the observed data by the method of simple trial and error. This method is explained in more detail in the following paragraph.

Driver's Table XIII contains the data for the determination of the limits of the effective period at 27°, which was estimated in the manner mentioned above to be 22 hours for males beginning at 57 hours and ending after 79 hours of larval life were passed at this temperature. The larvae in this case were transferred to 17°, at which temperature it was estimated from Driver's Table X that the effective period for males was 62 hours long extending from the 130th to the 192nd hour of larval life at this temperature. Flies developing from larvae transferred to 17° at the 61st hour at 27° had finished 4 hours of the effective period at 27°. This corresponds to 11.3 hours at 17°. The average number of facets which flies with such history show is given in Driver's Table XIII as 28.0. After trial and error it was estimated that about 1 facet was produced at 27°. The remainder, 27 facets, were produced in the remaining percentage of the period which is equivalent to 50.7 hours at 17°. Since males have on the average 29.0 facets at 17°, from these values then 2.0 facets are produced on this basis in 11.3 hours at 17°. Similarly it was estimated in this way that at 27° after 7 hours in the effective period there were 3 facets, after 9 hours 5.9 facets, after 13 hours 11.5 facets, after 17 hours 16 facets, after 20 hours 17.4 facets. The value of *K* at 27° was then determined for these dissected values and found to be 0.1696. (For all such determinations the use of Table LX, Robertson, 1923, greatly reduced the necessary calculations.)

It happens that 27° was the temperature to which larvae were transferred in experiments to determine the lengths of the effective period at 17°, 20°, and 22°. Similar determinations for *K* for the curve for 27° from the series of data (Driver's Tables X–XII) give the following values: 0.1615 for the transfer from 17°, 0.1479 for the transfer from 20°, and 0.1436 for the transfer from 22°. The close similarity of these four values for *K* makes one feel perhaps more confident in the approximation of the assumptions made to the actual situation. The average of the four values is 0.1557 which is taken as the final value of *K* for the reaction for males at 27°. A temperature of 17° was involved in two experiments. The values of *K* from these two cases were 0.0543 and 0.0593, giving an average of 0.0568. The remaining temperatures were involved only once in the transfer experiments. The limits of the effective period at 25° were not determined. This temperature was used however in the transfers from 15°. The length of the effective period was estimated by interpolation to be 24 hours for females and 23 hours for males. With these values then the value of K was determined from the values of the dissected data obtained from the experimental transfers from 15° to 25°.

It can be shown that the recombined calculated values are in satisfactory agreement with the observed values. The differences at only a few places are to any significant degree beyond the limits of what is statistically desirable. One of the worst fits is given by the comparisons to the data for males of Driver's Table IX: observed facet number, 19.9 ± 0.43, calculated, 18.79; observed, 21.10 ± 0.46, calculated, 20.74; observed, 24.18 ± 0.59, calculated, 25.4; observed, 26.70 ± 0.60, calculated, 29.84; observed, 31.34 ± 0.56, calculated, 31.5.

A comparison which is more nearly typical is given by the comparison of the recombined calculated results with the observed facet data for males of Driver's Table XII: observed, 17.58 ± 0.36, calculated, 18.28; observed, 18.20 ± 0.41, calculated, 18.7; observed, 18.71 ± 0.39, calculated, 19.17; observed, 19.02 ± 0.40, calculated, 19.6; observed, 19.58 ± 0.42, calculated, 19.92; observed, 20.34 ± 0.44, calculated, 20.2; observed, 20.77 ± 0.36, calculated, 20.6; observed, 21.20 ± 0.42, calculated, 21.1.

The results of the analysis carried out in this way are given in the accompanying Table I (see also Fig. 1). It can be seen from this table that with increasing temperature the duration in hours of the effective period decreases. A graph showing the relation is composed of two curves, concave upward, with a discontinuity in the neighborhood of 21°. The rate of the reaction as shown by k increases with increase in temperature and likewise shows a break near 21°. Since there are but three values on either side of the discontinuity it cannot be stated definitely whether k in its temperature relation conforms to the well known Arrhenius equation, $\ln k = \mu/RT$.

The facet number/temperature relation in the bar series conforms as an approximation to a simple exponential function $y = ae^{rt}$. For the ultrabar stock used in the present experiments r has the value -0.0443 for females and -0.0527 for males for the interval 15–27°. The first derivative of facet number with respect to temperature gives the facet decrease per degree. It may be considered as a measure of difference in growth rate. If this is the case then there should be some definite relation between the first derivative and k, the velocity constant of the facet-determining reaction, provided of course that the assumptions made for the determination of k are an approximation to the actual case. That there is such a relation can be seen from the data in Table I. For both males and females as k increases in geometric progression the first derivative decreases approximately in geometric progression, that is, the relation is approximately hyperbolic. From this result it seems safe to conclude that the use of the first derivative as a measure of the difference in growth and the underlying assumptions made to proceed with the analysis of the data for the determination of k of the facet-producing reaction are mutually confirmatory.

TABLE I

Summary of data on the temperature-effective periods and the constants of the sigmoid curve for the time course of facet determination in ultrabar males and females. The first derivative of facet number with respect to temperature is also given.

Temperature	Sex	Limits of effective period, hrs. of larval life	Limits of effective period, per cent larval life	Length of effective period, hrs.	Length of effective period, per cent larval life	A	K	$k \times 10^2$	$\frac{dy}{dt}$
°C.									
15	♀♀	190–288	32.98–50.0	98	17.02	26.5	0.0355	0.1340	1.18
15	♂♂	188–290	32.64–50.35	102	17.71	32.0	0.0330	0.1031	1.69
17	♀♀	127–192	38.48–58.18	65	19.6	24.6	0.0574	0.2333	1.08
17	♂♂	130–192	39.39–58.18	62	18.79	29.0	0.0568	0.1959	1.52
20	♀♀	108–156	45.00–65.00	48	20.0	21.5	0.0709	0.3298	0.95
20	♂♂	112–158	46.67–65.83	46	19.16	24.5	0.0705	0.2878	1.30
22	♀♀	83–112	48.54–65.50	29	16.96	19.5	0.1443	0.7400	0.87
22	♂♂	84–110	49.12–64.33	26	15.21	21.8	0.1132	0.5193	1.17
25	♀♀			24	17.80	17.5	0.1448	0.8274	0.76
25	♂♂			23	17.69	18.3	0.1605	0.8770	0.99
27	♀♀	56–77	50.00–68.75	21	18.75	15.5	0.1977	1.2755	0.69
27	♂♂	57–79	50.89–70.54	22	19.64	17.7	0.1557	0.8797	0.90

The facet-determining reaction may be considered further from two different aspects. First, in some sense it may be considered as a unit in itself and looked upon as one component of the whole developmental series, where it is coordinated in some functional relation to other component developmental processes. While it is interesting to know its length in hours yet from the point of view just expressed the question arises as to what is the relation which it bears to other similar processes for other organs and to the whole. Its duration in terms of the per cent of larval life which the period covers is approximately constant, about 18 per cent of the whole larval life. Its date, that is its temporal position in the developmental series, changes with change in temperature. It is earlier at the lower temperatures and later at the higher temperatures. For example, in females the effective period at 15° covers the interval from 33 to 50 per cent of the larval life and at 27° it begins at 50 per cent and extends to 68.8 per cent of the larval life (see Table I). From the point of view mentioned what we should especially like to do in this and similar cases is to relate the rate of the facet-determining process to that of other similar processes and to the rate of the development of the whole, such as might be obtained from data giving the growth of the larvae throughout their development and more particularly at the time of the facet reaction period. It is to be expected that a change in temperature affects such processes differentially (see Zeleny, 1923) so that the ratio of the relative growth rates will vary with the temperature.

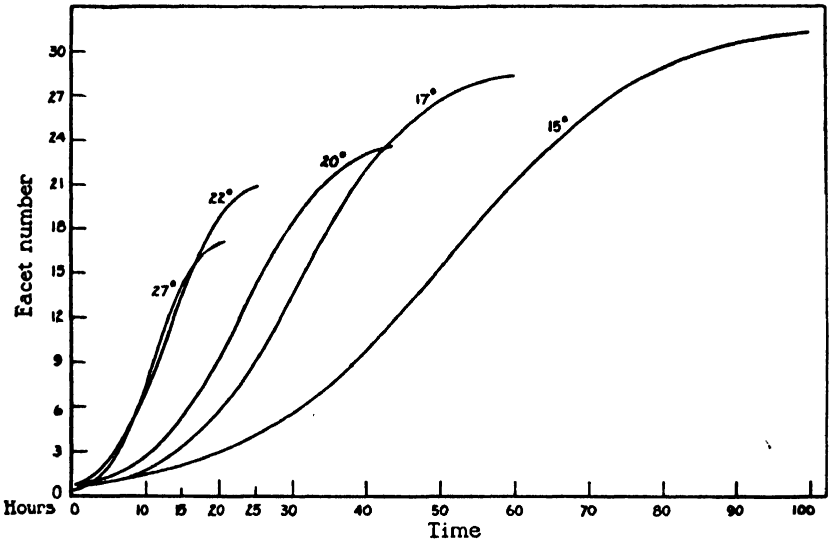

FIG. 1. Showing the course of facet determination at different temperatures in ultrabar males (data by Driver, 1931).

Some general notion of such relationships may be obtained from Fig. 2. Alpatov's data (1929, Tables 6–8) served as the basis for the curves for the growth in length of the larva during the three instars. Both axes have been reduced to a percentage basis and the effective periods for the males at the different temperatures superimposed at the appropriate places on the curves showing the percentage increase in larval length for the three instars. It can be seen that the effective periods for 15°, 17°, and 20° fall entirely or mainly in the second instar, while the effective periods for higher temperatures fall in the third instar. The conclusion suggests itself that the discontinuity found in

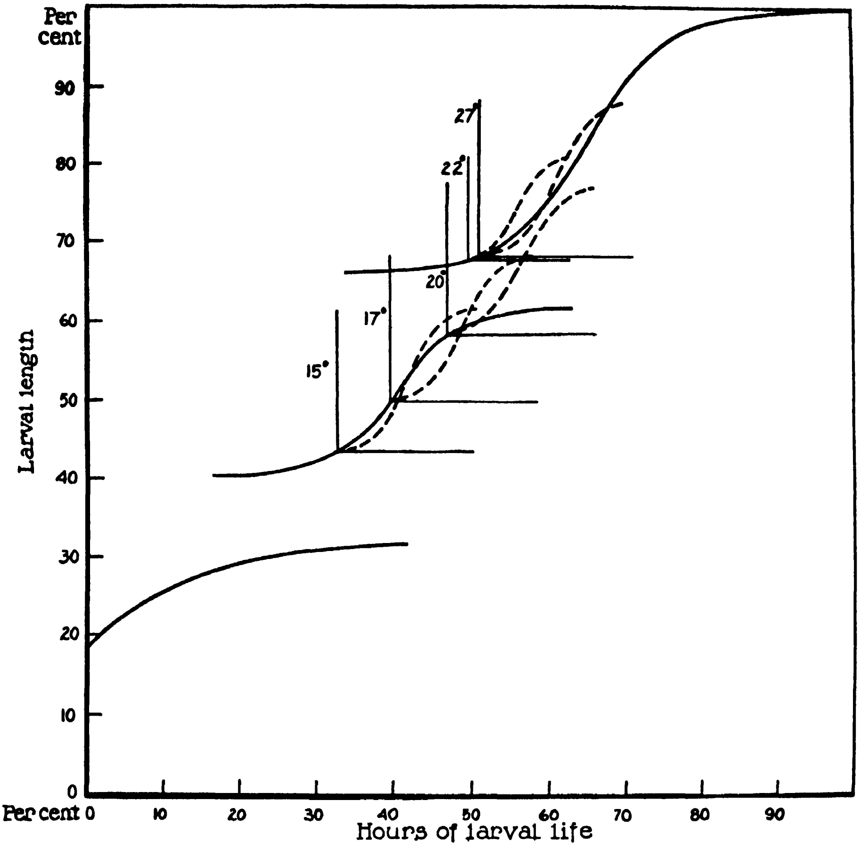

FIG. 2. The curves of Fig. 1 with each axis reduced to unity, that is taken as 100 per cent, are superimposed at their proper places upon the curves of growth showing the percentage increase in larval length for the three instars.

the value of k between 20° and 22° is causally related to the change in the relation of the effective period to the instars.

In the figure the effective period for each temperature has been plotted in a similar way with the abscissae representing percentages of the effective period and the ordinates percentages of the average number of facets at the different temperatures. The underlying idea here is that of the relative growth function, $y = bx^k$. One of the ways in which this function can be stated is as follows: If a variable x increases by a certain per cent, another variable y increases likewise by a certain fixed per cent. The ratio of the per cent increase in y to the per cent increase in x gives the value of k, the coefficient of growth partition. It is inadvisable to make any more direct application of the relative growth function to the data than can be grasped from the figure since the data available for growth in larval length are for 28° only and for a different stock. It can be seen, however, that k, the coefficient of growth partition, would almost certainly change with temperature and perhaps be constant for any particular temperature. There is an indication of a change in the coefficient of growth partition for those temperatures where the facet curve of Fig. 2 approaches its asymptote while the curve for increase in length is in a quite different section of its course. In regard to a value for k it can be seen from Fig. 2 that as the larval length, taken as x in the relative growth function, increases by about 1 per cent, the facet number (y) increases by about 5 to 7 per cent. Consequently for these variables k of the relative growth function has a value in the neighborhood of 5 to 7.

The facet-determining process may be considered, secondly, from the standpoint of the nature of the events which occur in the optic disc during the temperature-effective period. If the sigmoid curve is a true approximation for describing the course of the reaction then it may be said that differences between members of the bar series in regard to the process of facet determination do not represent merely developmental arrests of the process at some greater or lesser distance from a common upper asymptote, but the termination of the process is approached asymptotically. The same conclusion applies to the action of different temperatures on any particular member of the series, if ultrabar may be considered as typical of the entire series.

It is known from the work of Hewitt (1908) that in *Musca domestica* the optic disc (D) gives rise to ommatidia (O) and hypodermis (H) of the side of the head. This reaction which is equivalent at least formally both to a monomolecular reaction and to an embryonic segregation, may be symbolized as follows

$$D \rightarrow O + H$$

Is it this reaction which occurs during the effective period? Another possibility is the suggestion that during the effective period the optic disc is merely a center of mitotic activity with the embryonic segregation occurring instantaneously at the end of the period. Such an instantaneous reaction, at least from a chemical point of view, would require that the elements taking part in ommatidial production would be in the same state at that instant, so that the reaction (segregation) would take place with infinite rapidity. A third possibility would allow for the optic disc during the effective period to be both a center of mitotic activity and also the locus of an embryonic segregation.

On any of these views it would seem necessary to assume that at the beginning of the effective period there would already be a greater or smaller number of cells either to begin a cycle of mitotic activity or to participate in an embryonic segregation. Consequently one would expect the reaction of the effective period to be inaugurated by an initial flash productive of a greater or smaller number of facets. But if this is the case the consistent results presented above would imply that such an initial flash at the beginning of the period in ultrabar would not be extensive enough to obscure the sigmoid character of the main reaction.

SUMMARY

By a dissection of the data obtained by Driver on the effective periods at different temperatures in males and females of an ultrabar stock of *Drosophila melanogaster* it has been found that a symmetrical sigmoid curve satisfactorily describes the time course of the facet-determining reaction. Consequently the differences between members of the bar series in regard to this reaction do not represent merely developmental arrests of the process at some greater or lesser distance from a common upper asymptote, but the termination of the process is approached asymptotically. The velocity constant/temperature relation shows a discontinuity in the neighborhood of 21° which may be causally related to the change in the position of the effective period from the second to the third instar. The velocity constant apparently does not conform to the well known Arrhenius equation in its relation to temperature.

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THE REVERSIBLE INACTIVATION OF BACTERIOPHAGE BY BICHLORIDE OF MERCURY

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We have shown (1) that the inactivation of antistaphylococcus bacteriophage by 1:10,000 $HgCl_2$ (0.01 per cent or M/2720) at 22°C. in infusion broth adjusted to pH 7.6 proceeds according to the equation: $dP/dt = K[HgCl_2]\,[P_o - P_i]$ over the range studied, where P = [phage] in activity units, P_o = initial phage/ml., and P_i = phage/ml. inactivated in time t.

It was further demonstrated in these experiments that if the inactivated suspension were exposed to the action of H_2S when only some 5 per cent of the original phage remained active the initial titre of the phage suspension was restored. Control experiments indicated that the restoration of activity occurring upon precipitation of Hg^{++} ions was due not to any propagation of residual active phage but rather to a reactivation of the phage originally inactivated.

The question arose as to the influence of the time-concentration factor on the inactivation process; *i.e.*, whether prolonged exposure to Hg^{++} ions or the use of high $HgCl_2$ concentrations would irreversibly inactivate phage. The present experiments were conducted with this in mind.

The general procedure was to expose standard phage suspensions containing 1×10^{10} activity units/ml. (2) to the action of various concentrations of $HgCl_2$ for given lengths of time. An aliquot of each suspension was then titrated for residual active phage while the remainder was treated with H_2S to precipitate Hg^{++}. The excess H_2S was removed by thorough aeration and the phage concentration of the reactivated suspension determined. Details of the methods and the experimental controls employed are described in our earlier paper (1).

A. Inactivation

Table I is a summary of eleven experiments. It is evident from the results in Experiments 1–6 that inactivation progressing for long periods of time does not follow the previously recorded inactivation curve which holds for short periods. According to calculations based on the experimentally determined value of K for the equation $k = \frac{1}{t[\mathrm{HgCl_2}]} \cdot \ln \frac{P_o}{P_o - P_i}$ when $[\mathrm{HgCl_2}] = 1/10{,}000$ (0.01 per cent), all the phage should be inactivated at 4.4 hours. Actually, however, traces of active phage can be detected even after 268 hours. These residual concentrations are beyond the range of titration; for not less than 1×10^4 activity units/ml. can be measured accurately on this scale. It is not possible then to follow the remainder of the reaction quantitatively and it can merely be said that while the inactivation process fits the curve for a pseudomonomolecular reaction until the amount of active phage remaining is less than 0.5 per cent of $[P]_o$, the reaction beyond this point apparently proceeds more slowly than would be anticipated.

B. Reactivation

Reference to Table I shows that 100 per cent yields resulted when reactivation was performed even after exposure of phage to $\frac{1}{2}$ saturated $HgCl_2$ (2.8 per cent) for 216 hours. In certain earlier experiments, not reported here, we were unable to effect a 100 per cent reactivation if $[HgCl_2]$ were very high and found that to accomplish this end it was necessary to dilute the $HgCl_2$-phage mixture sufficiently (*e.g.* 1:1,000) before precipitating the Hg^{++}. When this precaution was followed the entire original quantity of phage could be recovered (*cf.* Experiments 9, 10, and 11).

C. Test for an Activator

It has been suggested that the phenomenon of phage inactivation and reactivation involves some hypothetical activator specific for phage rather than the lytic principle itself. Upon this basis it would be assumed that the reversible inactivation of phage represents a reaction between Hg^{++} and the activator, the phage being altogether unaffected by the bichloride. Against such a postulate are two facts, first that no one has demonstrated the existence of an activator for phage and, second, that the entire phenomenon of bacteriophagy is explicable as a reaction between a single substance, phage, and the bacterial cell. Accordingly, there would be no necessity at this time to complicate the facts further by picturing phage as consisting of two components. Certainly the tendency to analyze biological phenomena by invoking different reactants for each stage of a reaction has not produced happy results in the past.

TABLE I

Reactivation of Antistaphylococcal Phage after Exposure to $HgCl_2$ *by Precipitation of* Hg^{++}

All experiments in infusion broth at 22°C. pH 7.6.

No.	[$HgCl_2$]	Reactivated after	Initial [phage]	Residual [phage] after inactivation	[Phage] after reactivation	Phage recovered
		hrs.				*per cent*
1	1/10,000 0.01 per cent	24	5×10^9	5×10^7	4×10^9	80
2	1/10,000 0.01 per cent	48	5×10^9	3×10^5	5×10^9	100
3	1/10,000 0.01 per cent	48	5×10^5	$1 \times 10_5$	4.5×10^9	90
4	1/10,000 0.01 per cent	72	5×10^9	3×10^4	5×10^9	100
5	1/10,000 0.01 per cent	168	5×10^9	Trace	5×10^9	100
6	1/10,000 0.01 per cent	268	5×10^9	Trace	5×10^9	100
7	1/2,000 0.05 per cent	48	5×10^9	Trace	4.8×10^9	96
8	1/2,000 0.05 per cent	120	5×10^9	Trace	5.2×10^9	104
9	1/36 2.8 per cent	50	5×10^9	None	4.5×10^9	90
10	1/36 2.8 per cent	50	5×10^9	None	5×10^9	100
11	1/36 2.8 per cent	216	5×10^9	None	4.7×10^9	94

To seriously rule out the existence of an activator for phage is a formidable experimental task and for the present it seemed adequate merely to attempt its detection by certain simple obvious tests. These have been conducted by assuming that the hypothetical activator has a particle size different from that of phage or that it is more heat-resistant than phage.

Experimental.—Phage was filtered through an acetic collodion membrane (3) of the minimal pore size permitting it to pass without reduction in titre. If the activator particles were larger than phage one would expect them to be retained by the membrane. The primary filtration was followed by filtration with five separate 10 cc. amounts of broth and the membrane was then triturated in broth to put in suspension retained activator particles. This fraction was called No. 1.

Similarly phage was filtered through an acetic collodion membrane (3) of maximal pore size retaining phage completely. Should the activator be smaller than phage the filtrate (fraction 2) should contain it.

It has been shown (4) that a temperature of 60°C. acting for 1 hour will completely inactivate phage. Were the activator more resistant to temperature than phage it should survive this treatment. Accordingly phage was heated to 60°C. for 1 hour under the conditions specified in the earlier paper (4) and after cooling, it was designated fraction 3.

To test for the presence of an activator in the above three fractions phage was partially inactivated by exposing it to 0.01 per cent $HgCl_2$ for 0.4 hour. Samples for titration were diluted 1:1,000 and 1:10,000 with broth, in order to determine the residual concentration of "activated" phage. Identical samples were diluted 1:1,000 with each of the three fractions to be tested for the presence of the activator. If such an activator were present it would be expected to replace that destroyed by $HgCl_2$. However, the titration figures were identical in each case indicating the absence of an activator under the conditions obtaining in these tests.

DISCUSSION

The accepted mechanism of action of bichloride upon bacterial cells involves a twofold effect: first, a preliminary stunning of the growth function, and, second, with increasing time-concentration values, a profound lethal action. It has been demonstrated that in certain instances the primary toxic effect can be reversed by precipitation of the Hg ions (5). The second phase of the reaction, however, cannot be reversed.

This sequence of events apparently does not take place when $HgCl_2$ acts upon phage. That is, within comparable limits of concentrations and time the bichloride-phage reaction is completely reversible while the bichloride-bacterium reaction is only partially reversible. Unfortunately one difficulty presents itself in comparing available data concerning the two reactions; the reports on reversible disinfection of bacteria employ a qualitative criterion of survival in judging the efficacy of reversal procedures, and reversal is said to have occurred if any fraction of the treated bacterial suspension, no matter how small, is capable of growth. There is nothing to suggest in such end-point experiments that reactivation has taken place uniformly throughout the bacterial population, whereas in our phage experiments we not only have demonstrated the qualitative phenomenon of reversal but have shown that *all* the inactivated phage could be reactivated.

Admitting this incongruity of data, there is still sufficient analogy to make comparison interesting. For example, Gegenbauer (6) found that *S. aureus* cultures exposed to 1:2,000 $HgCl_2$ (0.05 per cent) contained viable survivors after 1.3 hours but were altogether non-viable after 2.3 hours. When exposure was followed by treatment with H_2S, survivors were present after 72 hours in $HgCl_2$ but not after 101 hours. Chick's results with *B. paratyphosus* (5) are of the same general order. With phage 100 per cent of the original lytic principle can be recovered after 120 hours exposure to the identical concentration of bichloride.

Even when the very resistant spores of *B. anthracis* are employed as the test organism and the same end-point technique is followed, bacterial protoplasm is found to be irreversibly damaged by high concentrations of Hg^{++}. Thus Müller (7) reported the following experimental data:

Anthrax Spores Treated with $HgCl_2$ at 37°C.

Concentration $HgCl_2$	Growth when antidote was added after	No growth when antidote was added after
per cent	*days*	*days*
0.1	9	12
1.0	7	9
2.0	7	9
3.0	6	7

In contrast phage can be reactivated with a 100 per cent yield after 9 days in 2.8 per cent of $HgCl_2$.

The experimental evidence then sets phage apart from bacterial protoplasm whether the latter is a vegetative cell or a resistant spore form, in that a single reagent, bichloride, acting upon these two substances appears to evoke fundamentally different reactions. It might be said, of course, that just as anthrax spores are more resistant to the final lethal action of $HgCl_2$ than vegetative cells in general, so phage is simply a still more resistant form of the same elementary protoplasm and that analogy is incomplete merely because we have not examined all existing forms of bacterial life from the standpoint of inactivation and reactivation. This conclusion is theoretically as tenable as another which could be drawn from the experimental data; namely, that the difference between the bichloride-bacterium reaction and the bichloride-phage reaction speaks for a significant difference between bacterial protoplasm and the substance we call phage. Here analogy would place phage with the enzymes, for there are recorded numerous instances of *complete* reactivation of enzymes after inactivation with a variety of ions (8–11).

In the light of present knowledge one cannot conclusively select one hypothesis to the absolute exclusion of the other. It can merely be said that if phage consists of small corpuscles of protoplasm, as some believe, it is a unique form of this substance, infinitely less susceptible to the toxic effects of $HgCl_2$ than the most durable forms so far investigated.

CONCLUSIONS

1. The complete inactivation of antistaphylococcal phage by $HgCl_2$ (2.8 per cent for 216 hours) can be reversed by precipitation of Hg^{++} with restoration of the phage to its original titre.
2. This behavior seems more compatible with the known properties of certain enzymes than with those of living protoplasm.

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THE KINETICS OF PENETRATION

VIII. Temporary Accumulation

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Potassium accumulates in many living cells, becoming much more concentrated inside than outside. This also happens in certain models[1] in which carbon dioxide is continuously supplied to the artificial sap to imitate its production by the living cell. In such models the concentration of potassium steadily increases up to a certain point and then remains stationary while the volume of the artificial sap continues to increase. This seems analogous to what happens in growing cells.

The nature of accumulation in such cases can be shown more clearly by setting up a system which is left to itself instead of being continually supplied with new material.

As an example we may cite a model in which 0.03 M KOH was placed in an outer aqueous phase *A* separated by a non-aqueous phase *B*, from an inner aqueous phase *C* containing 1.0 M HCl, which may be called artificial sap. Phase *B* consisted of 70 per cent guaiacol plus 30 per cent *p*-cresol which substances may be collectively designated H*G*. KOH reacts with H*G* to form K*G* and passes as such through *B* to *C* where it reacts with HCl to form KCl. The result is the same as though KOH passed as such, so that we may speak of KOH as passing through *B* to *C*.

A typical experiment (No. 123) was made as follows: Model I was used.[1] Phase *A* contained at the start 2 liters of 0.03 M KOH, phase *B* 1100 cc. of G. C. mixture (70 per cent guaiacol plus 30 per cent *p*-cresol) which was saturated with water, and phase *C* contained 100 cc. of 1.0 M HCl. Phases *A* and *B* were stirred mechanically, phase *C* by bubbling a stream of air. The temperature varied from 21–25°C. From time to time phase *C* was removed, its volume determined, and a sample taken for analysis. The total acidity was determined by titration with standard alkali, the concentration of chloride ion was determined by titration with standard silver nitrate, potassium thus being found by difference. Values for the potassium concentration so obtained checked satisfactorily with those found by our customary procedure.[1]

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[1] Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931–32, **15**, 667.

We have KOH on one side of the non-aqueous layer and HCl on the other. They tend to mix by passing through this layer and forming KCl in *A* and in *C*. But in a given time so much more KOH than HCl passes through *B* that most of the KCl is formed in *C*, where the concentration of potassium becomes much greater than in *A*. This, however, is only temporary for when the system comes to equilibrium the composition of *A* and *C* will be identical since all the substances present can pass through the non-aqueous layer.

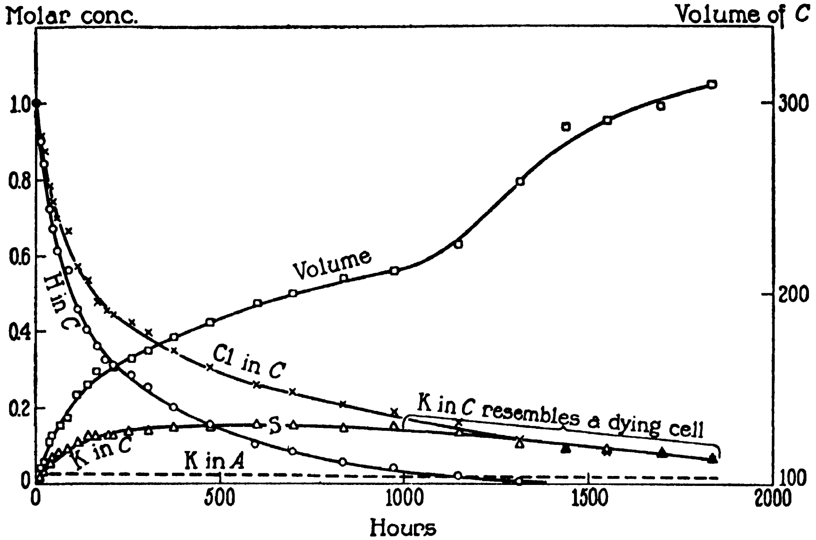

FIG. 1. Curves showing that the concentration of potassium in *C* reaches a maximum (at *S*) and then falls off. The concentration of KOH in *A*, and of HCl in *C*, falls from the start. In this experiment (No. 123) *A* contained at the start 0.03 M KOH, *B* was guaiacol plus *p*-cresol, and *C* contained 1.0 M HCl at the start. Volumes of *C* are given in cubic centimeters.

The behavior of potassium is seen in Fig. 1.[2] Its concentration in the artificial sap (in *C*) increases until it is much greater than in the external solution (in *A*). It reaches a maximum at *S* and then declines.[3] At equilibrium it will be the same in *A* and *C*. The decline is analogous to what happens in a dying cell and equilibrium corresponds to death.[4]

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[2] In this, as in the other figures, the curves are drawn free-hand to give a rough fit.

The rapid inward movement of potassium at the start depends on two things, (1) KOH reacts with the guaiacol and *p*-cresol to form organic salts which may be collectively called K*G* and treated as one since their behavior is very similar. (2) K*G* has a relatively high partition coefficient[5] (defined as the concentration in the non-aqueous divided by that in the aqueous phase) and in consequence a relatively high concentration gradient in *B*, as the result of which its diffusion[5,6] through *B* is relatively rapid. (As explained in previous papers[5,6] it moves through *B* chiefly in molecular form.)

When K*G* reaches the artificial sap it is changed to KCl according to the scheme

$$\mathrm{K}G + \mathrm{HCl} = \mathrm{KCl} + \mathrm{H}G$$

and H*G* is retained in *B*. The result is the same as if KOH penetrated and reacted in the sap according to the scheme

$$\mathrm{KOH} + \mathrm{HCl} = \mathrm{KCl} + \mathrm{H_2O}$$

We may use this scheme as more convenient and state that potassium will tend to enter as long as the ionic activity product (K) (OH) is greater in *A* than in *C*.

As KCl has a relatively low partition coefficient in *B* its concentration gradient remains low and it moves outward very slowly. Eventually, however, as its concentration in *C* rises, its outward movement becomes great enough to offset the entrance of K*G* so that the concentration of potassium becomes practically stationary for the time being (*S*, Fig. 1).

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[3] The concentration declines for two reasons, (1) water moves from *A* to *C*, and (2) KCl moves from *C* to *A*: there is also a movement of HCl in the same direction which continues as long as the pH of *A* is greater than that of *C* (which is still the case at 1551 hours).

These movements, of course, influence the volumes. At the start the volume of *A* is 2 liters: this declines as water moves into *C*. The volume of *C* which starts at 100 cc. increases and at 1551 hours is 290 cc. This is to be expected since the osmotic pressure is higher in *C* than in *A*.

[4] This might mean a Donnan equilibrium in case the cell contained ions unable to pass out through the cell wall. Otherwise the internal and external solutions would eventually become identical.

[5] Osterhout, W. J. V., Kamerling, S. E., and Stanley, W. M., *J. Gen. Physiol.*, 1933–34, **17**, 445, 469.

[6] Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529.

This temporary stationary state corresponds to the permanent steady state in those models in which the concentration of K*G* in *A* is kept constant[5,6] and HCl is replaced by CO_2 in *C*, the concentration of the latter being kept constant. We then find (Fig. 2) that the accumulating substance (which is $KHCO_3$ instead of KCl) increases until its concentration is much higher in *C* than in *A*. In consequence water enters *C* and this continues until the steady state is reached in which

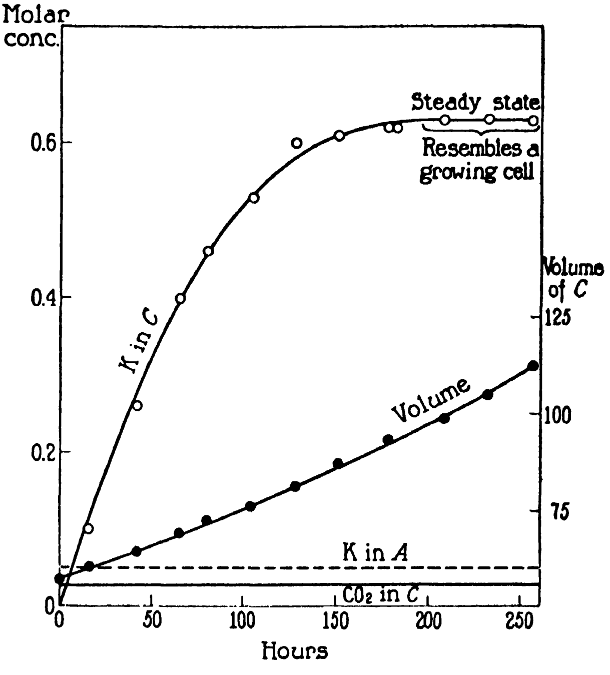

FIG. 2. The concentration of potassium rises to a fixed value; that of potassium in *A* and of CO_2 in *C* remains constant. In this experiment (No. 64 of previous papers[1,5,6]) *A* contained at the start 0.05 M K*G* and this was kept approximately constant by a continuous flow, and *C* contained at the start distilled water; CO_2 was bubbled through *C* during the entire experiment; *B* was guaiacol plus *p*-cresol. Volumes of *C* are given in cubic centimeters.

water and electrolyte enter in a fixed ratio and the volume of C continues to increase while its composition remains approximately constant.[7] Consequently the potassium curve rises to a certain level at which it remains. If the supply of CO_2 falls off the pH of C rises and the curve declines.

The steady state appears to be analogous to what we find in growing cells. For example, when we add to the sea water in which *Valonia* is growing[8] a small amount of NH_3 it enters and there is a rapid accumulation of NH_4Cl which reaches a steady state in which its concentration remains approximately constant.

It would seem that in the cell, as in the model, accumulation depends on the fact that the permeability to the accumulating substance is greater for the form in which it enters than for the form in which it goes out (*e.g.* greater for NH_3 than for NH_4Cl and greater for KG than for KCl). This depends chiefly on the partition coefficients and diffusion constants.[5,6,9]

Before leaving this subject let us glance for a moment at the effect of injury. This may produce a twofold effect, (1) increase of permeability, (2) lessened accumulation. The total amount of electrolyte taken up in a given time will be influenced by these factors and in the injured cell may be greater than normal or less, depending somewhat on the length of the interval of time chosen. A dead *Valonia* cell might for a short time take up more electrolyte than a living one but in the end the living cell would take up more.

Let us now consider the energy relations. We may write

$$F_A = F_o + 2.3\, RT \log (\mathrm{K}_o)\ (\mathrm{OH}_o)$$

and

$$F_C = C_o + 2.3\, RT \log (\mathrm{K}_i)\ (\mathrm{OH}_i)$$

where F_o is the thermodynamic potential of 1 mole of KOH in the standard state, F_A and F_C are the thermodynamic potentials of KOH in *A* and *C* respectively: $(K_o)(OH_o)$ and $(K_i)(OH_i)$ are the ionic activity products outside (in *A*) and inside (in *C*) respectively.[10,1] Hence

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[7] This is Experiment 64 of a preceding paper (see footnote 1). See also footnote 5.

[8] Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 301. The ammonia appears to enter as NH_3 rather than as NH_4^+.

[9] See Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, **35**, 983, ff., 1002.

$$\Delta_{\mathrm{KOH}} = F_A - F_C = 2.3\ RT \log \frac{(K_o)\ (OH_o)}{(K_i)\ (OH_i)}$$

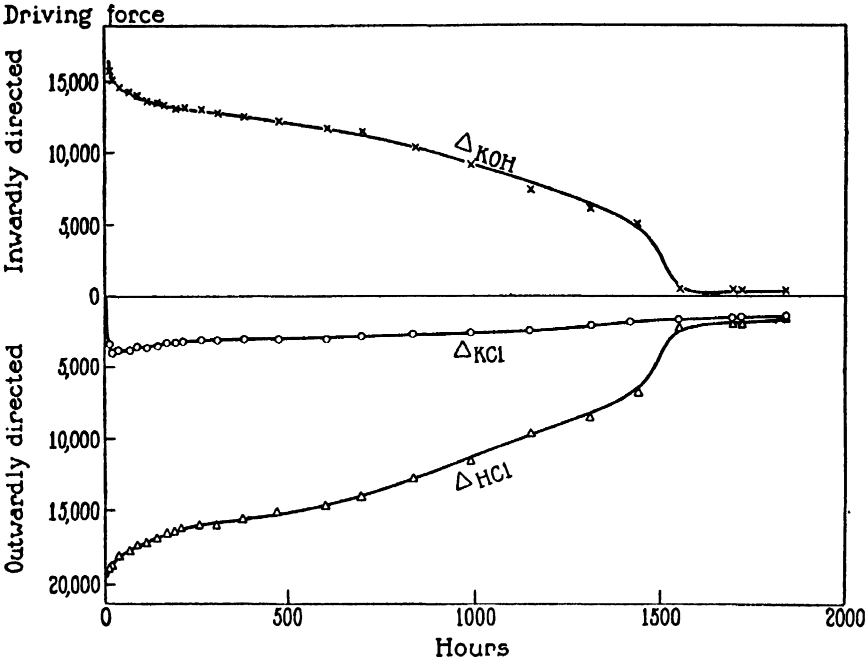

FIG. 3. Shows changes in the model (Experiment No. 123; see Fig. 1) in respect to the following: Δ_{KOH}, the driving force which causes KOH to move inward (*i.e.* from *A* to *C*); Δ_{HCl}, the driving force which causes HCl to move outward (*i.e.* from *C* to *A*); and Δ_{KCl}, the driving force which causes KCl (the accumulating substance) to move outward (this passes through a maximum). The ordinates for Δ_{KOH} represent the value of

$$\Delta_{\mathrm{KOH}} = 2.3\ RT \log \frac{(K_o)\ (OH_o)}{(K_i)\ (OH_i)};$$

the ordinates for Δ_{HCl} represent the value of

$$\Delta_{\mathrm{HCl}} = 2.3\ RT \log \frac{(H_i)\ (Cl_i)}{(H_o)\ (Cl_o)}$$

(the equation for Δ_{KCl} is similar). We put $R = 1.988$ and $T = 296°$.

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[10] Cf. Lewis, G .N., and Randall, M., Thermodynamics, New York, McGraw-Hill Book Co., 1923.

It is evident that Δ_{KOH} represents a driving force directed inward (*i.e.* causing KOH to move from *A* to *C*) and Δ_{HCl} and Δ_{KCl} represent driving forces directed outward. To make this evident in the graph (Fig. 3) Δ_{KOH} and Δ_{HCl} are plotted in opposite directions.

We see that Δ_{KCl}, starting at zero, rises and then falls. The fall could be prevented if HCl were kept constant in *C* and if KOH in *A* were continuously renewed by a flow of solution which at the same time removed KCl from *A*.[11] This is evident from Fig. 4 which shows

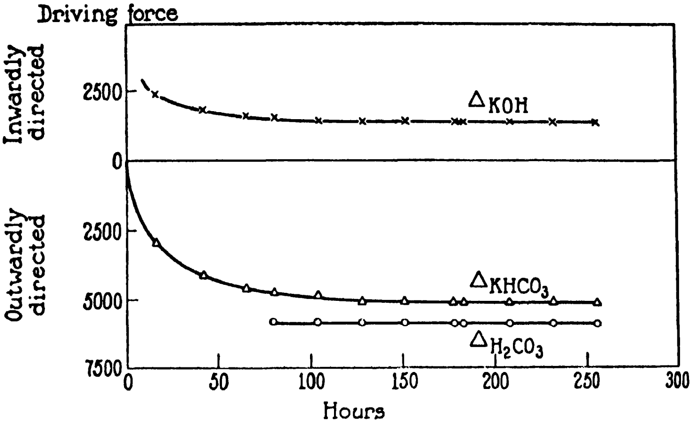

FIG. 4. Shows behavior in the model (Experiment No. 64; see Fig. 2) of the following: Δ_{KOH}, the driving force which causes KOH to move inward (*i.e.*, from *A* to *C*); $\Delta_{H_2CO_3}$, the driving force which causes H_2CO_3 to move outward (*i.e.*, from *C* to *A*); and Δ_{KHCO_3}, the driving force which causes $KHCO_3$ to move outward. After Δ_{KHCO_3} becomes constant the volume of the artificial sap in *C* continues to increase (which seems to be analogous to what happens in growing cells). Δ_{KOH} and $\Delta_{H_2CO_3}$ would show more change if KOH and CO_2 were not continuously renewed (this differs from the experiment shown in Fig. 3 in which KOH and HCl were not renewed). The ordinates for Δ_{KOH} represent the value of

$$\Delta_{KOH} = 2.3\,RT \log \frac{(K_o)\ (OH_o)}{(K_i)\ (OH_i)};$$

the ordinates for $\Delta_{H_2CO_3}$ represent the value of

$$\Delta_{H_2CO_3} = 2.3\,RT \log \frac{(H_i)\ (HCO_{3i})}{(H_o)\ (HCO_{3o})}$$

(the equation for Δ_{KHCO_3} is similar). We put $R = 1.988$ and $T = 296°$.

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[11] This case would differ from that illustrated in Fig. 1 in that HCl moving into *A* produces relatively little effect since the solution in *A* is continually renewed.

an experiment of this sort in which, however, CO_2 was substituted[12] for HCl in *C*, its concentration being kept constant.

The calculation of Δ made it necessary to take account of the activities of K^+ and Cl^-. Although these are not known an attempt was made to approximate[13] them as follows: the activities of K^+ and Cl^- were regarded in all cases as equivalent to those in a solution of KCl of the same ionic strength, on the assumption that in such a solution the activities of K^+ and Cl^- are equal. The ionic strength in *A* is practically equivalent to the concentration of K^+ and in *C* to the concentration of Cl^-.

The activity of KCl was taken from the tables of Harned.[14] The concentrations in *A* of K^+ and Cl^- and the activities of H^+ and OH^- were in some cases estimated by graphic interpolation.

In the experiment shown in Fig. 3 energy is furnished by the reactions $KOH + HG = KG + H_2O$ and $KOH + HCl = KCl + H_2O$, and also by differences in osmotic pressure in *A* and *C*. In the experiment shown in Fig. 4 the chief sources of energy are the reaction $KG + H_2CO_3 = KHCO_3 + HG$, and likewise the continuous addition of K*G* and CO_2 to the system. There are also differences in osmotic pressure.

DISCUSSION

During the first part of the experiment potassium tends to go in as KOH (since $(K_o)(OH_o) > (K_i)(OH_i)$) and as K*G* (since $(K_o)(G_o) > (K_i)(G_i)$), and at the same time to go out as KCl (since $(K_i)(Cl_i) > (K_o)(Cl_o)$). These opposing tendencies do not balance until K_i becomes much greater than K_o: hence potassium accumulates. The reason is that much less KCl than K*G* moves through *B* in a given time because the partition coefficient of the latter is relatively high.

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[12] The value of $\Delta_{H_2CO_3}$ falls off somewhat because the concentration of CO_2 increases somewhat in *A* because CO_2 moves from *C* into *A* a little faster than it is removed by the flow of liquid in *A*.

It will be noticed that no points are given on the graph in Fig. 4 for $\Delta_{H_2CO_3}$ in the early part of the experiment. This is because no determinations were made of CO_2 in *A* during the early part of the experiment.

[13] A rough approximation is all that can be attempted as individual ion activities cannot be ascertained. See Guggenheim, E. A., *J. Phys. Chem.*, 1929, **33**, 842; MacInnes, D. A., in Cold Spring Harbor symposia on quantitative biology, 1933, **1**, 190.

[14] Harned, H. S., in Taylor, H. S., A treatise on physical chemistry, New York, D. Van Nostrand Company, Inc., 2nd edition, 1931, **1**, 769, 772.

This illustrates very clearly that in addition to the driving force and the diffusion constant we must also take account of the partition coefficients. Since K*G* and KCl are weak electrolytes in *B* it is the diffusion constants of the molecules with which we have to do. As K*G* has the larger molecule it probably has the smaller diffusion constant, yet moves more rapidly than KCl through *B* because its partition coefficient and in consequence its concentration gradient is greater. Similar principles apply to living cells (where potassium may unite with a constituent *X* of the protoplasm to form K*X*).

It is evident that, other things being equal, accumulation will increase as the pH of the sap decreases, since the products[15] $(\text{K}_i)(\text{OH}_i)$ and $(\text{K}_i)(G_i)$ will be kept lower and consequently the driving force of K*G* and KOH will be greater. Hence anything that checks the production of acid by the cell may be expected to check accumulation and likewise growth. Conversely a rise of the external pH may be expected to have the opposite effect. This accords with experience, as discussed elsewhere.[9]

This model recalls the situation in *Valonia* (and many other living cells) where potassium accumulates chiefly as KCl. It has been suggested[16,9] that in this case potassium enters as KOH and forms K*A* in the sap (where *A* is an organic anion). In some plants it accumulates as K*A*[9] but when HCl exists in the external solution it will tend to enter and displace the weaker acid H*A* (if this be carbonic it can readily escape): hence potassium may accumulate to a greater or less extent as KCl.

SUMMARY

A model[17] is described which throws light on the mechanism of accumulation. In the model used an external aqueous phase *A* is separated by a non-aqueous phase *B* (representing the protoplasm) from the artificial sap in *C*. *A* contains KOH and *C* contains HCl: they tend to mix by passing through the non-aqueous layer but much more KOH moves so that most of the KCl is formed in *C*, where the concentration of potassium becomes much greater than in *A*. This accumulation is only temporary for as the system approaches equilibrium the composition of *A* approaches identity with that of *C*, since all the substances present can pass through the non-aqueous layer. Such an approach to equilibrium may be compared to the death of the cell as the result of which accumulation disappears.

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[15] What applies to one of these products applies also to the other since under the conditions of the experiment G^- stands in a constant relation to OH^- (Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529. Similar considerations may apply to a considerable extent to living cells.

[16] Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, **24**, 234; *J. Gen. Physiol.*, 1930–31, **14**, 285; *Biol. Rev.*, 1931, **6**, 369.

[17] *Note Added to Proof.*—Our attention has been called to a paper by Teorell (Teorell, T., *Skand. Arch. Physiol.*, 1933, **66**, 225) describing temporary accumulation.

During the earlier stages of the experiment potassium tends to go in as KOH and at the same time to go out as KCl. These opposing tendencies do not balance until the concentration of potassium inside becomes much greater than outside (hence potassium accumulates). The reason is that KCl, although its driving force be great, moves very slowly in *B* because its partition coefficient is low and in consequence its concentration gradient in *B* is small. This illustrates the importance of partition coefficients for penetration in models and in living cells. It also indicates that accumulation depends on the fact that permeability is greater for the ingoing compound of the accumulating substance than for the outgoing compound.

Other things being equal, accumulation is increased by maintaining a low pH in *C*. Hence we may infer that anything which checks the production of acid in the living cell may be expected to check accumulation and growth.

This model recalls the situation in *Valonia* and in most living cells where potassium accumulates as KCl, perhaps because it enters as KOH and forms K*A* in the sap (where *A* is an organic anion). In some plants potassium accumulates as K*A* but when HCl exists in the external solution it will tend to enter and displace the weaker acid H*A* (if this be carbonic it can readily escape): hence potassium may accumulate to a greater or less extent as KCl.

Injury of the cell may produce a twofold effect, (1) increase of permeability, (2) lessened accumulation. The total amount of electrolyte taken up in a given time will be influenced by these factors and may be greater than normal in the injured cell or less, depending somewhat on the length of the interval of time chosen.

THE VISUAL ACUITY AND INTENSITY DISCRIMINATION OF DROSOPHILA*

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I

Problem and General Method

The visual functions of many animals vary with the prevailing light intensity to which they are subjected. In general the visual capacities are poor at low illuminations and become increasingly better as the intensity rises (Aubert, 1865; Koenig and Brodhun, 1889; Koenig, 1897; Hecht, 1924; and Hecht and Wolf, 1929). In recent years certain ideas have been proposed which offer an explanation of this capacity for variation in the visual system and which link this capacity with other apparently unrelated properties of photoreception (Hecht, 1931). Given a photosensory organ composed of a number of discrete receptor elements, each containing a particular photochemical system, then the various data of vision may as a first approximation be described in terms of commonly accepted properties of photochemical and chemical reactions and in terms of the distribution with respect to their sensibility to intensity of similar elements in a population.

Up to the present, the human eye has been the visual system for which most data have been available. However, a distinct difficulty in the quantitative derivations and comparisons of the various sets of data for the human eye is that even when they have been secured with the same eye the conditions of measurement have not been the same, so that both the similarities and the differences have often had to be discounted and their meaning obscured. The various measurements with *Mya* and similar animals, though fairly extensive (Hecht,

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* A preliminary report of these measurements was presented at the XIV International Physiological Congress in Rome in September, 1932.

1931), do not involve functions such as visual acuity which are particularly interesting in this connection. We therefore determined to measure some of these functions in another animal whose vision is significantly different from our own so as to furnish the basis for an independent description of its underlying physiological structure. In this paper we record such measurements of visual acuity and of intensity discrimination as they are influenced by the intensity.

A previous success in the measurement of visual acuity with the bee (Hecht and Wolf, 1929) led us to use the common fruit-fly, *Drosophila melanogaster*, which in addition to the genetic uniformity also possessed by the bee, has the advantages of ease of culture and year-round availability. Obviously the use of such an animal demands the development of a special procedure for measuring visual response. This has already been accomplished for the bee and can be used with modifications for the fly. The method depends on the reflex response given by an animal to a movement in its visual surroundings. Presented with a visual field composed of a definite pattern, an animal can obviously respond to a movement of this pattern only when it is able to resolve the essential elements of the pattern. The composition of the pattern may then be varied to obtain a measurement of either visual acuity or of intensity discrimination.

The simplest pattern is a series of stripes. For visual acuity the stripes may be varied in width and the intensity determined at which they are just resolved. For intensity discrimination the relative intensities of the alternating stripes may be varied and the minimum difference in intensity determined which, for a given stripe width, will just elicit a response at a series of selected intensities. We arranged our apparatus so that both measurements could be made on a single fly in the same set-up and under identical conditions.

II

Nature of the Reflex Response

The response of the fly presents several interesting aspects. A fly is allowed to creep freely along the horizontal length of a narrow glass cell placed parallel to an illuminated, vertically striped plate constituting its entire visual field. With the striped plate at rest the fly usually creeps back and forth from one end of the 7 cm. cell to the other. If now the striped plate is moved in the direction in which the fly is creeping, the fly stops, creeps backward for a few millimeters, turns around, and rapidly runs off in the opposite direction. This behavior is almost diagrammatic. By moving the plate repeatedly back and forth, it is possible to keep the fly revolving about any point in the cell.

It is simple to show that the behavior of the fly is not acquired during its first essays at motion. We allowed several pupae to emerge in complete darkness, each in an experimental cell. The response of the flies during their very first exposures to light was just as characteristic and clear as that of older flies raised in the light. A measurement of the threshold for a response to a given stripe made with one of these flies gave a value of 1.8×10^{-2} millilamberts; the measurement occupied 3 minutes during only a small fraction of which the fly was actually illuminated. 8 days later its threshold was 1.5×10^{-2} millilamberts, an agreement well within the daily variability of the animals. Between these two measurements the fly had been exposed daily to the light from an open window. Evidently the response of *Drosophila* to moving patterns is an inherited, complicated reflex.

Almost all animals with eyes perform such directed reactions when presented with a movement in their visual environment (Lyon, 1904; Garrey, 1905; Hadley, 1906; Demoll, 1909; Doflein, 1910; Loeb, 1918; Schlieper, 1927; Hecht and Wolf, 1929; Grundfest, 1931; Schulz, 1931). The response is either *with* the direction of the environmental displacement, or *against* the displacement. Thus fish under certain conditions follow a moving pattern (Grundfest) as do certain arthropods (Schlieper), whereas bees (Hecht and Wolf) and *Drosophila* move against the background motion.

The animals which move with the motion of the background are fish, aquatic insects, crabs, and hovering insects, which maintain relatively stationary positions for some time even in a moving medium. Very likely they accomplish this by optically fixating some portion of their visual field and adhering to it even if they have to swim or fly against the current. On the contrary, the animals which move against the displacement in their visual field are bees and flies which move in a relatively stationary environment. When they fly or creep, their visual environment usually passes by them. Their response when their visual field suddenly begins to overtake them is then concerned with so orienting themselves that the visual environment assumes its characteristic motion past them.

It is to the point that the one animal which has been studied under both conditions may go with or against a movement in its visual field depending on whether the animal itself is fixed or free to move. The honey bee when it creeps freely always goes against any movement of its visual environment. Confronted with a series of moving stripes, the change in direction of creeping of the bee results from the bending of the head and thorax in the new direction opposite to the stripe movement. On the other hand when the bee is fixed in position and confronted by a similar movement of stripes, the head and thorax characteristically follow the movement (Schlieper).

The main significance of these responses for us is that they may be used as a tool in the quantitative study of the visual capacities of animals. In the present experiments, the response of the fly was used merely to indicate that the fly resolved the particular pattern presented to it under the given conditions. The response is so vigorous and clear-cut that even at threshold conditions it is unmistakable. Actually at these threshold conditions the fly does not leap backward and turn about; rather it stops when the stripes are moved in the direction in which it is creeping, and starts again when the stripes are moved in the opposite direction. This was the constant response used as end-point in all the measurements to be described.

We made no special effort to control precisely the speed of the plate movement used in evoking the reaction. However, this motion was always sufficiently slow so that any complication by fusion of the stripes is out of the question.[1] To obtain a sharp response it is not necessary to move the striped plate more than just perceptibly faster than the movement of the fly itself.

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[1] This is the difficulty with the work of Graham and Hunter (1931) who in using this method for measuring the visual acuity of humans found that a moving pattern yielded markedly different results from a stationary pattern. One of us (G. W.) with the help of Dr. Harry Grundfest repeated enough of Graham and Hunter's measurements to be certain that such discrepancies disappear when the plates are moved with the velocity which we habitually use in these experiments. This was confirmed personally by Dr. Graham, who saw these measurements. It emerged that in the work of Graham and Hunter the pattern had been moved very much more quickly,—so quickly indeed that fusion occurred. This high rate of motion of the pattern completely accounts for the aberrant results obtained by these authors.

III

Apparatus and Procedure

The relation which visual acuity and intensity discrimination both bear to the prevailing intensity may be measured in two ways. One may set a given intensity and determine by trial with the animal what the visual acuity or the intensity discrimination corresponding to it is; or one may choose a pattern corresponding to a given visual acuity or select a given intensity difference and by trial with the animal determine the intensity at which the resolution of the pattern takes place or the intensity difference is recognized. With intensity discrimination we used both methods; with visual acuity only the latter.

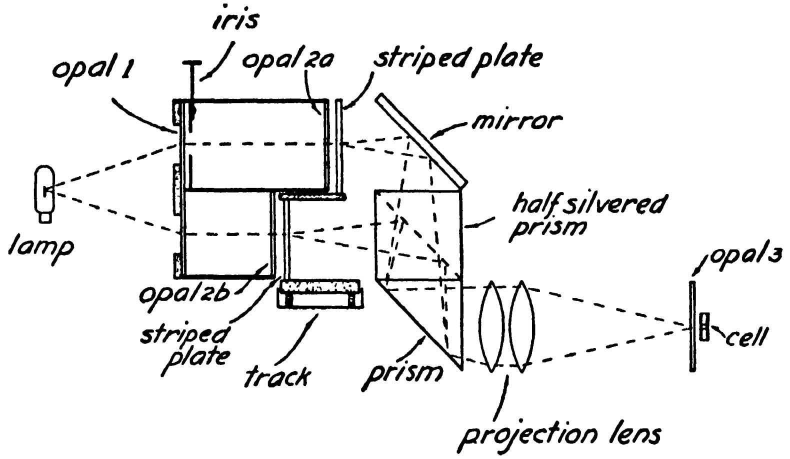

FIG. 1. First apparatus for measuring intensity discrimination

The apparatus used for the first method of measuring intensity discrimination consists essentially of a movable set of vertical stripes separated by interspaces of the same width, the whole being so arranged that the illumination of the stripes and of the interspaces may be controlled independently. It may be understood by reference to Fig. 1.

The light from a 500 watt concentrated-filament Mazda lamp falls on two separated, and light-insulated portions of an opal plate (opal 1), thus forming two secondary sources of illumination, an upper and a lower. The lamp is kept in one dark room, the rest of the apparatus in another; the wall between the two contains two openings for the light to reach the two portions of opal 1, which is in immediate contact with the openings in the wall. The intensity falling on opal 1 is varied by placing the lamp at different fixed distances from the openings. At a given position of the lamp, the intensity of the lower secondary source remains fixed, and illuminates an opal plate (opal 2 *b*) immediately in front of which is a series of opaque vertical bars separated by equal sized transparent spaces. The upper secondary source similarly illuminates an opal plate (opal 2 *a*) in front of which is a duplicate bar and space arrangement. The intensity falling on opal 2 *a* can be varied by means of an accurate iris diaphragm immediately in front of the upper secondary source which controls its radiating area. A fixed diaphragm in front of the lower secondary source so adjusts its radiating area that with the iris wide open the illumination on opal 2 *a* is just perceptibly greater than on opal 2 *b*, even though the latter is nearer opal 1.

The light from the two series of bars and spaces, after reflection by a mirror and prism, is focussed with a projection lens on a third opal screen. The optical paths of the light from the two sets of stripes to the screen are of identical length and composition; hence the two are projected in simultaneous focus. The two sets of stripes are mounted on the same heavy brass carriage which moves on roller bearings along a track perpendicular to the plane of the drawing in Fig. 1. The relative positions of the two sets of stripes are so adjusted that in the projection on the final opal screen (opal 3) the image of the bars of one falls exactly in the clear spaces of the other. The result on the final screen is a movable series of alternating, illuminated stripes whose relative intensities may be controlled by the iris diaphragm at the upper secondary source. When the iris diaphragm is slightly closed, the two sets of stripes are of equal brightness and the field is uniform. When the iris diaphragm is completely closed, every other stripe is at zero illumination and the field is a series of black bars separated by equally wide, illuminated interspaces. At any intermediate position of the iris, the bars may take on any intensity value between zero and that of the interspaces. As already indicated, the intensity of the interspaces may be set at any desired value by regulating the position of the Mazda source. The width of each stripe on the final pattern occupies a visual angle of 85°; as will be apparent later, this is well above the largest visual angle required for the lowest visual acuity of the fly.

The fly is placed in a rectangular glass cell in front of the final opal screen on which the moving pattern is projected. The interior of the cell is about 2 mm. high, 3 mm. wide, and 70 mm. long. The fly, which is broader than it is tall, always walks upright or inverted in the cell, never on the sides. The fly thus always turns one eye full toward the pattern. The fly is observed through a cylindrical reading lens mounted parallel to the cell.

The measurements are made as follows. A fly is put into the apparatus and allowed to walk freely when the light is on. By means of the iris diaphragm, the intensities of the two sets of stripes are equated. Motion of this uniform field produces, of course, no response. The brightness of the variable stripes is now progressively decreased by decreasing the iris diaphragm, and the animal subjected to the moving pattern with each small diminution. A point is soon reached when the animal responds to the movement, indicating that it can distinguish between the intensities of the alternating stripes.

The apparatus for the second method of measuring intensity discrimination is much simpler than for the first method. Here the animal is presented with a plate so constructed that its alternating stripes transmit a fixed ratio of intensities, and the prevailing intensity is adjusted until the fly responds to a movement of the plate. Fig. 2 makes the arrangements clear. Light from a 500 watt concentrated-filament Mazda lamp in one dark room falls on an opal glass plate (opal 1) which covers an opening in a rectangular box mounted on the wall of the dark room. The opposite end of the box opens against a hole in the wall leading into an adjoining dark room. Immediately against the hole is another opal glass (opal 2) which forms one wall of a second rectangular box of which the opposite wall is a third opal glass plate (opal 3). The intensity of the light falling on opal plate 3 is controlled (*a*) discontinuously by placing the lamp at three selected distances from opal 1, and by removing opal 1, and (*b*) continuously for the intervening steps by means of the accurate iris diaphragm in front of opal plate 2. The illumination on opal plate 3 can be accurately and continuously varied over a range of 6 logarithmic units of intensity.

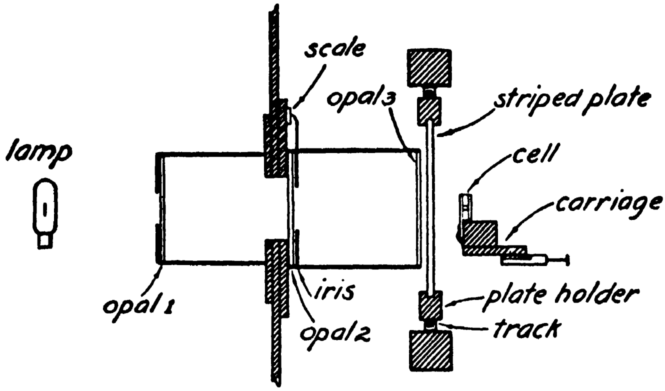

FIG. 2. Second apparatus for measuring intensity discrimination. This set-up also serves to measure visual acuity.

Immediately in front of opal 3 is the striped plate, mounted in a carriage which slides easily along brass tracks. A plate is composed of translucent bars and equally wide, clear interspaces. For all the plates the width of the bars and spaces is the same, and the clear interspaces are the same. The plates differ in the density of the bars, so that each plate represents a pattern of stripes whose alternating elements have a fixed ratio of light transmissions. The plates were prepared by photographically enlarging on Eastman Process plates a striped pattern accurately engraved by Max Levy and Company of Philadelphia. The Levy plate consists of equally wide, alternating opaque and clear stripes, such as were employed in the visual acuity work with the bees (Hecht and Wolf). By varying the time of exposure and keeping all conditions of lighting and development constant, a graded series of stripe densities were obtained. The six plates which we used were calibrated for the transmissions of the stripes and the clear spaces with a Koenig-Martens spectrophotometer using light of 500 mμ.

The measurements are made by setting for each plate an intensity at which motion of the plate produces no response. The intensity is then gradually raised by small steps until the characteristic threshold reaction of the animal is elicited. Knowing the intensity of the clear spaces and the relative transmissions of the bars and clear spaces, we have a measure of the difference in intensity required at a given intensity for the fly to respond to the stripes.

We began with the first apparatus, but soon abandoned it for the second, which we adopted because of its greater simplicity, and because we could also use it for measuring visual acuity. All that is required for determining visual acuity with the second apparatus are plates of proper density and size of stripe. We prepared photographically a series of striped plates, using Eastman Process plates and Eastman special hydroquinone developer. They were all enlargements of the accurately made Levy plate previously mentioned. The size of the stripes was varied, but the exposures were complete. In this way we secured plates with stripes of a very high degree of opacity, transmitting certainly less than 1/10,000 of the incident light, and with interspaces which were almost perfectly clear. The transmissions of the clear spaces were nevertheless measured and an appropriate correction applied to the intensity.

The procedure for making the visual acuity measurements is similar to that for intensity discrimination. For each plate an intensity is set at which a motion of the plate evokes no response. The intensity is then gradually raised and the fly tested until an intensity is found at which it just gives its characteristic response. Knowing the distance of the fly from the plate and the width of the stripe, the visual acuity is merely the reciprocal of the visual angle in minutes. The distance of the fly from the plate was kept constant for each series of measurements, though it was not the same for all the series. It is of the order of 15 mm. and is measured from the center of the eye.

The flies used in all the measurements here recorded were selected from a homozygous wild-type stock and were grown in the cornmeal-agar-molasses medium, seeded with yeast, used by Morgan and his coworkers. In our final measurements only females were used, because they crawled more slowly and steadily than the males. Each female was usually supplied with a male, and the pairs were quartered in individual vials containing 2 per cent agar in Pasteur's medium seeded with yeast. In this way a fly could be kept active for 3 weeks and longer.

IV

RESULTS

1. Intensity Discrimination.—With the first apparatus we made measurements of the intensity discrimination of seven flies during

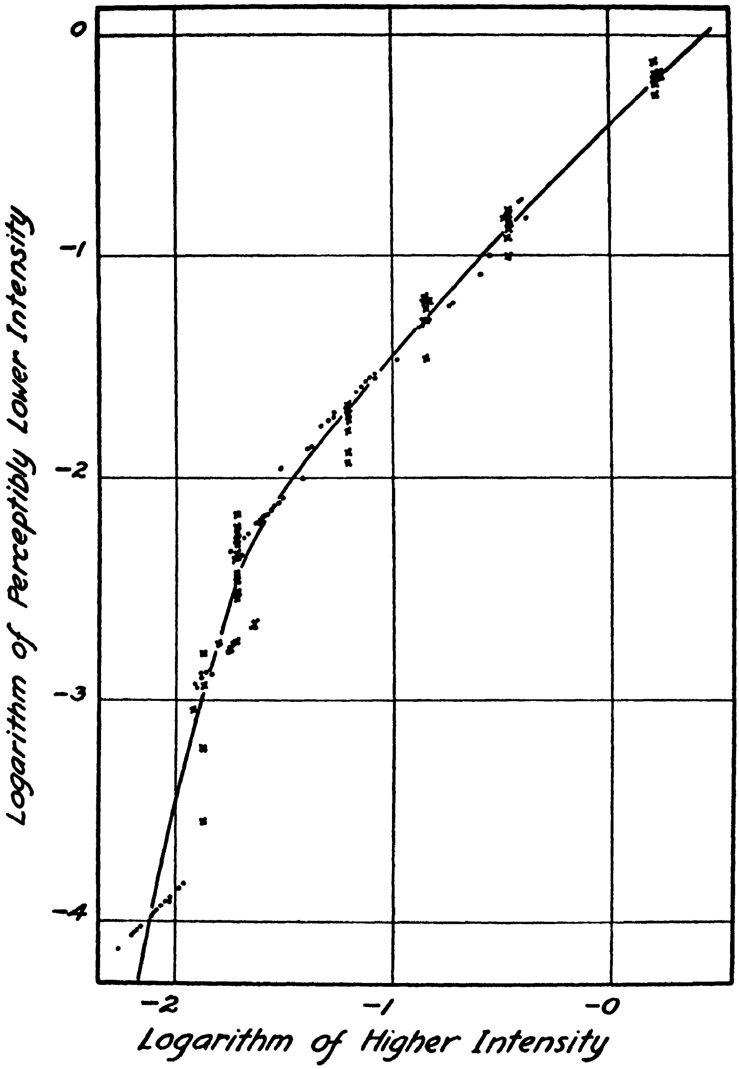

FIG. 3. Intensity discrimination of *Drosophila*. Each measurement with 24 flies is recorded. The crosses were secured with the first apparatus; the dots with the second apparatus.

October and November of 1930; with the second apparatus we measured seventeen flies between February and June of 1931. Each measurement with each fly is shown in Fig. 3, where those made with the first apparatus are crosses and those with the second apparatus are dots. It is apparent that the results from the two sources are the same, and that the data are homogeneous. The 113 measurements have been combined in the obvious groups into which they fall in the plot in Fig. 3, and have been averaged. These averages are given in Table I. The curve drawn through the data in Fig. 3 is made to pass through these average points, and it is plain that the averaged data are a real representation of the individual measurements.

TABLE I

Intensity Discrimination of Drosophila

No. of readings	Higher intensity $(I + \Delta I)$	Lower intensity (I)	Perceptible difference (ΔI)	$\frac{\Delta I}{I}$	$\frac{\Delta I}{I + \Delta I}$
	millilamberts	*millilamberts*	*millilamberts*		
16	0.00773	0.000104	0.00763	73.33	0.987
12	0.0137	0.00109	0.0126	11.57	0.920
24	0.0186	0.00300	0.0156	5.20	0.839
12	0.0269	0.00706	0.0198	2.81	0.736
13	0.0533	0.0165	0.0368	2.33	0.690
6	0.0753	0 0269	0.0484	1.80	0.643
9	0.139	0.0532	0.0858	1.61	0.617
14	0.333	0.132	0.201	1.52	0.604
7	1.62	0.647	0.973	1.50	0.601

The data show that at the lowest intensities, for the fly to recognize the pattern, the higher intensity has to be about 100 times as strong as the lower. As the intensity increases, the just perceptibly lower intensity increases at first much more rapidly than the higher, then the two increase at about the same rate until the ratio of higher to the just perceptibly lower intensity becomes about 2.50, which value is maintained up to the highest intensities. This is shown by the fact that the plot in Fig. 3 rapidly approaches a straight line with a slope of 45°.

In order to be certain that the ratio of the two perceptibly different intensities undergoes no further change as the intensity increases, we tested flies at intensities up to 1000 millilamberts. Our first apparatus varies the ratio continuously, but unfortunately cannot achieve high intensities. The second apparatus can reach high intensities, but gives ratios in discrete steps only. Using the second apparatus we therefore tested flies at three intensities to each of three plates whose stripes transmit light in the ratios of 1.84, 2.17, and 2.77 respectively. The data for Fly 10c are given in Fig. 4. The measurements made in the usual way are marked with circles. The responses of the fly to the highest three intensities are shown by a minus sign when it failed to respond, by a plus sign when it responded clearly, and by a combination of the two when its behavior was doubtful.

Fig. 3 and Fig. 4 show that intensity discrimination in *Drosophila* begins to be effective at a prevailing brightness of 0.008 millilamberts. At this value, intensity discrimination is extremely poor; but in the short space of half a logarithmic unit the intensity discrimination improves at a tremendous rate so that it reaches very nearly its maximum value, which then remains constant for 4.5 log units up to the highest intensities obtainable in the measurements.

It should be pointed out that even these high intensities are not really high for *Drosophila.* We give the intensities in millilamberts, which are brightness units for our eyes. Our photometric measurements represent the effectiveness of light in terms of the efficiency of the spectrum, which for our eyes with a 500 watt lamp is maximal between 560 and 570 mμ. The maximum effectiveness of the spectrum for *Drosophila* is at 360 mμ at which point the light is easily 100 times more effective than at 560 mμ (Bertholf, 1932). Considering the comparatively trifling amount of light of 360 mμ which a 500 watt lamp emits, and the trifling amount which the various opal glasses transmit, a brightness of 1000 millilamberts thus secured must be a fairly low intensity for *Drosophila.* It will therefore be important to extend these measurements of intensity discrimination using ultra-violet light.

A practical consequence of this situation for our present measurements is that a photometric reproduction of a given brightness in the present apparatus as made with visible light and with our eyes need not necessarily represent a similar amount of effective energy for *Drosophila* in the ultra-violet. Indeed we often did find variations in threshold which are very likely due to this factor. In combining the measurements of many animals as in Fig. 3 and also later in Fig. 7, we compensated for these occasional changes in threshold by shifting the data equally along both intensity axes to bring them into conformity. Since the intensity is plotted logarithmically, the procedure is simple and introduces no difficulties.

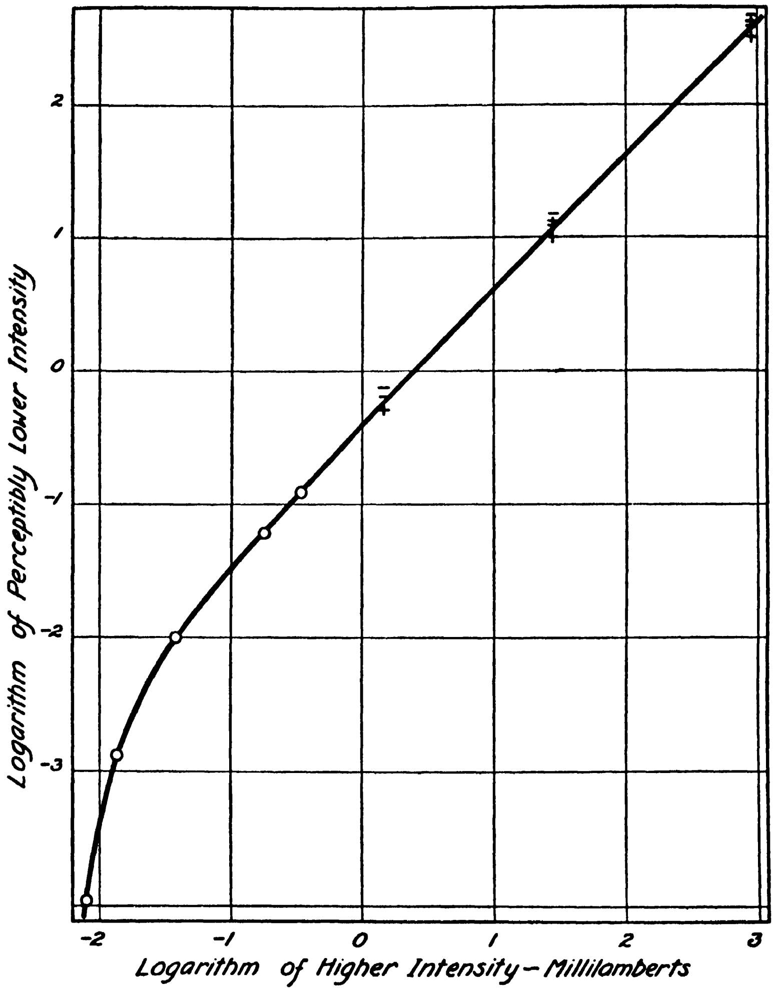

Fig. 4. Intensity discrimination of Fly 10 c to show that even at the highest illuminations intensity discrimination remains at its maximum and does not deteriorate.

In Table I and in Figs. 3 and 4 the measurements are given in their simplest and most direct form; namely as the two intensities which when placed side by side in stripes are just discriminated by the flies as indicated by their response to the movement of the pattern formed by these intensities. Following common procedure one may call the lower intensity I and the higher $I + \Delta I$, the difference between them being ΔI. We have computed $\Delta I/I$, and have plotted its values against log I in Fig. 5. In the present instance, this method of describing intensity discrimination overemphasizes the events at the very lowest intensities. A value of ΔI which is 80 times as large as I itself tells only that I is probably below or very near the threshold of visibility. In particular, the plot of $\Delta I/I$ against log I fails to bring out what is apparent from the direct data themselves (Figs. 3 and 4), that there is a sharp change in intensity discrimination at about log

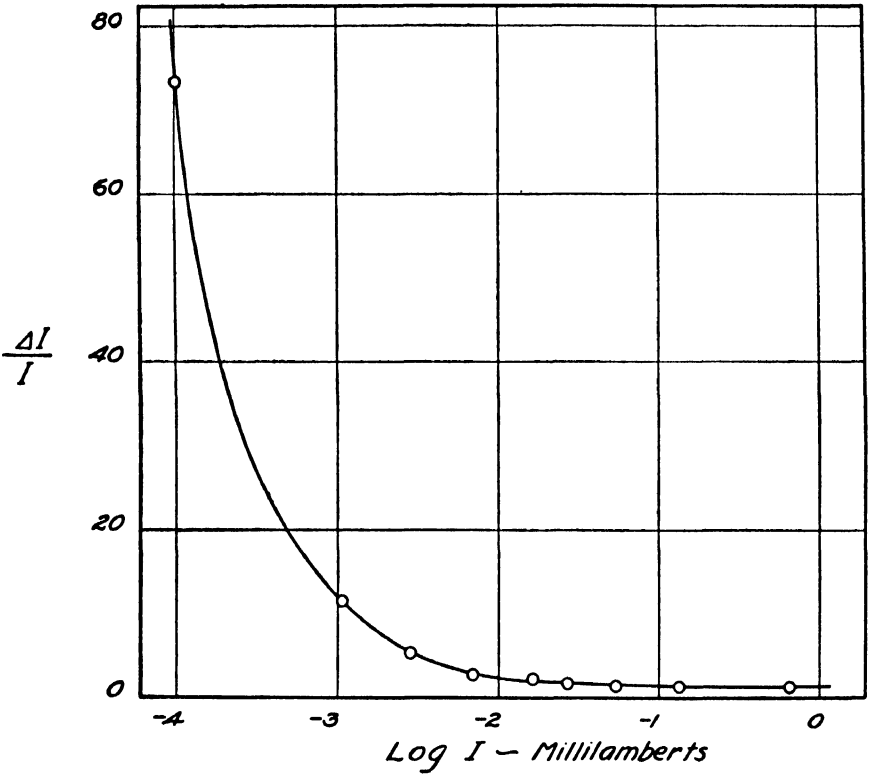

FIG. 5. The average data of intensity discrimination plotted as $\Delta I/I$ against log I. The fraction $\Delta I/I$ remains minimal at the highest illuminations.

$(I + \Delta I) = -1.7$ and that below log $(I + \Delta I) = -2.1$ intensity discrimination is practically non-existent.

A method adopted by many workers in photometric practice is to plot $\Delta I/(I + \Delta I)$ against log $(I + \Delta I)$. In the human eye where the difference between I and $(I + \Delta I)$ is very small, it makes little difference which of the two methods is used. But in the fly the difference is tremendous. Fig. 6 shows the data of Table I in this form, and indicates that this function is much more expressive of the real way in which the data behave. From Fig. 6 it is clear that below a value of log $(I + \Delta I) = -2.1$ for an intensity to be discriminated by the fly as lower than the prevailing intensity, it practically has to be extinguished,—which is the fact.

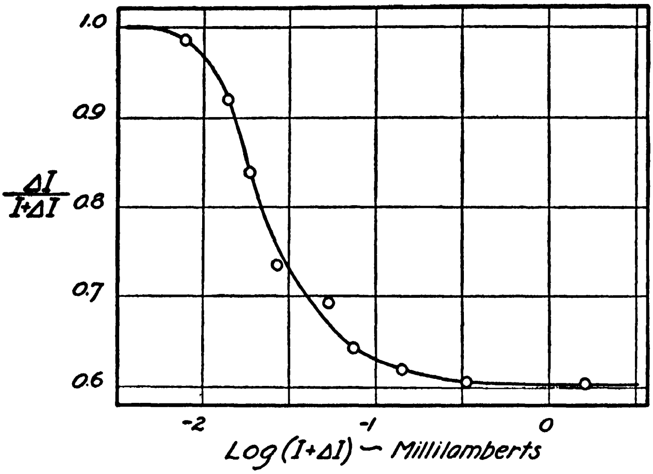

FIG. 6. The average data of intensity discrimination plotted as $\Delta I/(I + \Delta I)$ against log $(I + \Delta I)$. The function begins at about -2.1 and continues to improve steadily, showing no decline.

Whichever of the three ways one records the measurements, the fact remains that intensity discrimination for *Drosophila* changes first very rapidly and then more slowly over a small range of intensities above the threshold, and then reaches a constant value which is maintained as the intensity continues to increase. A similar condition holds for the bee's intensity discrimination as recently measured by Wolf (1933). The measurements of Koenig and Brodhun (1889) supported more recently by Lowry (1931), and by Houstoun and Shearer (1930), for the human eye show no constant value for the higher intensities; instead the intensity discrimination increases, and then decreases as the intensity rises. The same is apparently true for the clam (Hecht, 1924). The older data of Aubert on the human eye do not show this fall, and unpublished measurements by Mr. Jacinto Steinhardt of our Laboratory indicate that this fall at high intensities disappears under proper conditions of measurement.

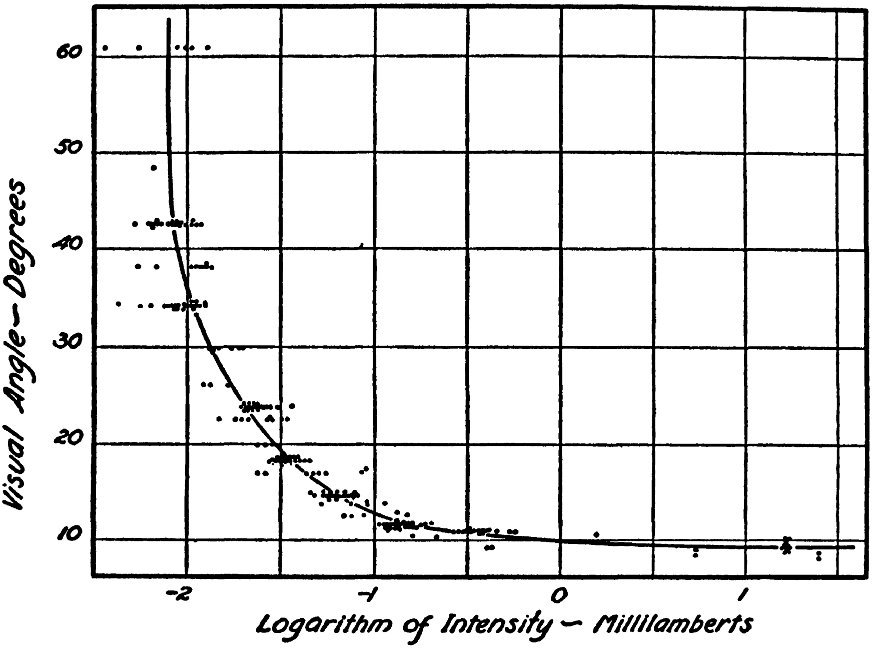

FIG. 7. The relation between intensity and the angular distance occupied by the stripes to which the fly can just respond. Each measurement with 32 flies is recorded.

2. *Visual Acuity.*—We measured the relation of visual acuity to illumination in twenty-four flies in November, 1929, and in eight more flies between March and August of 1931. Each measurement for each fly is given in Fig. 7. The ordinates are the actual visual angle subtended by the just visible stripe. The averages of the 220 measurements are recorded in Table II, and the curve in Fig. 7 passes through these average values. It is apparent that the measurements for the various animals form a consistent description of the phenomenon.

Graphically the data are best represented as visual acuity against the logarithm of the illumination, following the usual practice of defining visual acuity as the reciprocal of the just resolvable visual angle measured in minutes of arc. Fig. 8 shows the averaged data of Table II plotted in this way. There are several points to be made with regard to the data. Of these the most obvious is that visual acuity increases with the logarithm of the illumination in a sigmoid manner, already familiar from the data on the human eye, and on the bee eye. At the lowest intensities the visual acuity of *Drosophila* does not decrease continuously with the decrease in intensity, but instead stops quite sharply at an intensity corresponding to a brightness of 0.008 millilamberts. No matter how large the stripes are, the animals do not respond to them until this intensity is reached. This is made evident in Fig. 8 by the vertical line at this intensity, and was apparent in every animal which we tested for this purpose. This is related to the fact, obvious from Fig. 3 and Fig. 6, that at this intensity for another intensity to be recognized as perceptibly lower it must be practically extinguished.

TABLE II

Visual Acuity of Drosophila

No. of readings	Intensity	Visual angle	Visual acuity × 10^4
	millilamberts	*degrees*	
6	0.00794	61.08	2.73
20	0.00800	42.90	3.89
30	0.00966	35.33	4.72
10	0.0142	28.54	5.84
32	0.0234	23.30	7.15
24	0.0343	18.24	9.14
10	0.0511	16.58	10.05
20	0.0627	14.45	11.53
10	0.0908	13.19	12.64
25	0.141	11.69	14.27
20	0.378	10.73	15.53
13	14.6	9.28	17.95

The maximum visual acuity achieved by *Drosophila* is 0.0018, a value about 1/1000 that of the human eye, and 1/10 that of the bee's eye. This maximal value had to be obtained by a modification of the usual method as already described. The response of a fly to stripes depends on the size of the stripes, the distance of the fly from the stripes, and the intensity of the light. The usual method fixes the distance of the fly, presents it with a series of plates having each a fixed size of stripe, and measures the intensity required for the fly to respond to each stripe. To determine the maximum visual acuity in this manner requires the size of the stripe to be continuously variable—a difficult thing to achieve in practice. We therefore adopted the procedure of choosing a stripe of approximately the correct size, fixing a high intensity, and measuring the distance at which the fly must be in order just to respond to the movement of the stripes.

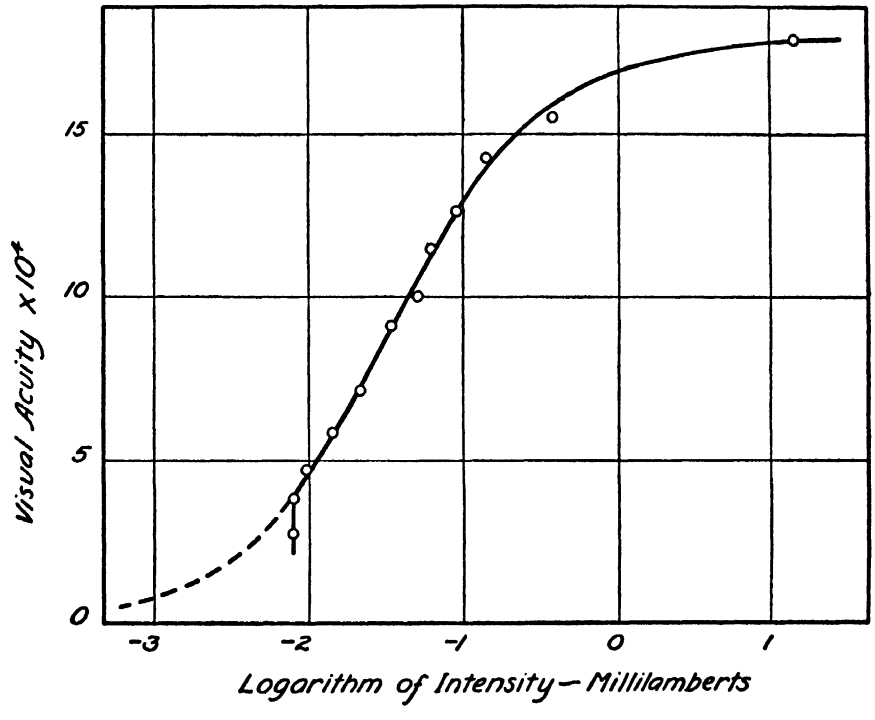

FIG. 8. The averaged data of Fig. 7 plotted as visual acuity against the logarithm of the intensity. The function starts abruptly at −2.1, below which the flies do not respond to stripes no matter how large they are.

The procedure of varying the distance of the fly from the test object may influence visual acuity by changing the brightness, and may complicate matters in the same way as the curious but unexplained effect of distance on human visual acuity first found by Aubert and Foerster (Aubert, 1865) and recently emphasized by Freeman (1932). Neither of these can be very serious for our measurements because the distances involved are 2 or 3 mm. Nevertheless we made special measurements to determine whether any such effects are present, varying the distance about 20 mm. Taking two plates with stripes 6.3 mm. and 1.27 mm. wide, we placed them 25.8 mm. and 5.21 mm. from the fly respectively. These both correspond to a visual acuity of 0.0012. Then we measured the threshold intensities of sixteen flies to a movement of these stripes, and secured as averages 4.36 and 4.07 respectively for the two plates. Similarly two plates having stripes 6.3 mm. and 2.84 mm. wide and at 21.5 mm. and 9.69 mm. from the fly (visual acuity = 0.0010) gave average intensity thresholds for the same sixteen flies as 1.66 and 1.86 respectively for the two plates. The differences between the two plates in each case are obviously negligible, and are opposite in direction in the two cases. The units of intensity here given do not correspond with the others previously given because we used a violet monochromatic filter in these measurements; according to Koenig (1897) the distance effect in the human eye is most prominent in the blue and violet, and we wished to make the test extreme. Therefore the determination of the maximum visual acuity by the distance method introduces no new variables, and the value 0.00180 for this maximum for *Drosophila* is the real value.

The maximum visual acuity of the human eye and the bee's eye is associated with the size of the structural units of the receiving elements (Müller, 1826; Ramon y Cajal, 1894; Exner, 1891; Best, 1911). In man the maximum value approximates the distance between foveal cones, though under special conditions it seems possible to increase the maximum performance (Hartridge, 1922; Anderson and Weymouth, 1923). In the bee the minimum perceptible visual angle (0.9°–1.0°) as determined physiologically (Hecht and Wolf) corresponds with the smallest angles (also 0.9°–1.0°) subtended by the ommatidia in the central portion of the eye as measured anatomically (Baumgärtner, 1928).

The ommatidial angles in the eye of *Drosophila* have not been adequately measured (Johannsen, 1924). We therefore prepared for this purpose many sections of eyes using essentially Baumgärtner's technique. This consists of rapidly fixing the heads in hot water, carefully running them through the alcohols, staining with eosin, imbedding in celloidin, preparing sections 20μ thick with a sliding microtome, clearing the sections with cedar oil, and mounting them in Canada balsam. We made photomicrographs of some of our best preparations, and on the mounted pictures we measured the angles between adjacent ommatidia. A thread stretched between two needles was passed through the axis of each ommatidium. The needle pricks were connected with a line and the angle between adjacent axis-lines measured.

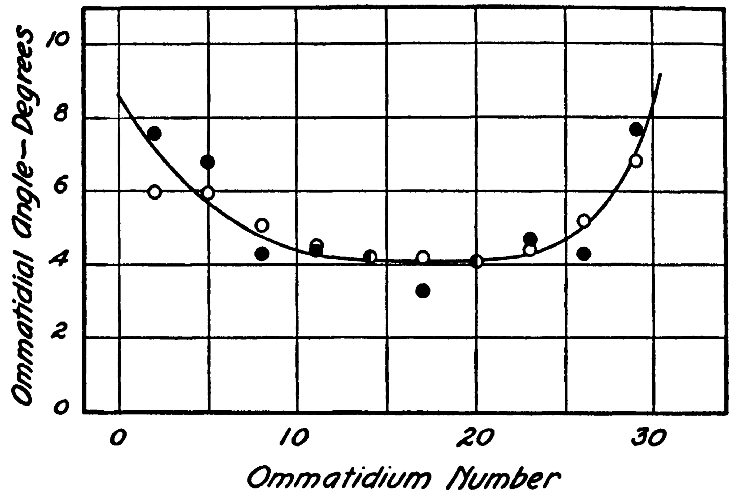

FIG. 9. The distribution of ommatidial angles in the left (open circles) and right (solid circles) eyes of *Drosophila* (Animal Iiii). The angles were measured for groups of three ommatidia, and the average ommatidial angle for each group is assigned to the middle ommatidium of the group.

The results are fairly irregular but, as Fig. 9 shows, quite adequate for the purpose. The largest section of the eye contains 31 ommatidia. The middle 16–18 of these show a constant angular separation of about 4.2°. At both ends the angular separation rises sharply to about 8°; measurements at the ends are uncertain due to the pronounced curvature of the ommatidia in these regions. The central region of the eye with its ommatidial separation of 4.2° is thus the place of sharpest vision, much as in the bee's eye. The average maximum visual acuity which we found experimentally for *Drosophila* corresponds to an angle of 9.28°, which therefore includes about two ommatidia instead of one.

This difference between the physiologically achieved and anatomically expected resolving power may mean that the neural paths of the ommatidia are interconnected, and that they therefore cannot act as individuals but as connected groups. However, we are inclined to ascribe it to another cause, namely the small number of ommatidia present in the eye as a whole. To distinguish a pattern, a certain minimal number of elements must be stimulated. This number is apparently a small fraction of the total population of retinal elements in the eye of man or of the bee. In the fly where the total number of elements is much smaller than in the human eye or in the bee eye, it probably represents a considerable proportion of the retinal population, and the group of units called into play to register a single stripe thus transcends the boundaries of a single line of elements. This idea is supported to a certain extent by the observation that homozygous bar-eye females, the eyes of which contain only 4–5 elements in the widest horizontal section, do not respond to the motion of the stripes at all.

Perhaps the best support for this idea comes from the experiments with the bee's eye in which parts of the eye were painted out. In the bee's eye it was found (Hecht and Wolf) that the maximum visual acuity coincided very well with the minimum angular separation between ommatidia, which shows that the individual elements act independently. Yet in an experiment in which the anterior half of each eye was painted out, the visual acuity at all intensities dropped to about 0.6 of its normal value, even at the maximum. Since the unpainted residue of the eye still contained elements having the original minimum angular separation, the drop in maximum visual acuity must be due to the decrease in the total number of elements acting in the eye.

V

Comparisons

A comparison between the two visual functions studied in *Drosophila* brings out the significant fact that the two functions begin and end at about the same intensities. As Fig. 8 shows, visual acuity begins to increase at an intensity whose logarithm is -2 and accomplishes most of its change in about 2 log units; the maximum visual acuity is not reached for about 1 log unit more, but this final change is very slow and not very large. Essentially the same thing is true of intensity discrimination. Fig. 3 and Fig. 6 show quite clearly that this function, after beginning at an intensity whose logarithm is -2, accomplishes most of its change in about 2 log units. Its maximum capacity is reached in about 1 log unit more, and this final change is slow and not very large.

The recently published measurements of the intensity discrimination of the bee by Wolf show that a similar relation exists between visual acuity and intensity discrimination for the bee. Visual acuity in the bee (Hecht and Wolf) begins to increase perceptibly with intensity at an intensity corresponding to $\log I = -1.0$, and accomplishes nearly all of its range at $\log I = 1.0$, though the small increase to the maximum visual acuity continues till after $\log I = 2.0$. The same range is covered by intensity discrimination. According to Wolf's data, $\Delta I/I$ begins to vary effectively at about $\log I = -1.5$ and accomplishes most of its range at about $\log I = 1.0$; its lowest value is reached after about one more log unit.

It would be well if a similar comparison of the two functions could be made for the human eye, but the existing measurements were made under such different conditions that it is not possible to do so with any certainty. Koenig's visual acuity data (Koenig, 1897) cover about the same range as his intensity discrimination data (Koenig and Brodhun, 1889), that is, between 8 and 9 log units; but the precise way in which the two functions vary has been called into doubt by Lythgoe's measurements of visual acuity (Lythgoe, 1932), and by unpublished measurements of intensity discrimination by Mr. Jacinto Steinhardt in our own Laboratory. For the present, therefore, it is well to omit discussion of them.

A comparison of the maximum values for intensity discrimination and visual acuity in the three species is of interest. The minimum value of $\Delta I/I$ for *Drosophila* is 1.5; for the bee it is 0.25 (Wolf); and for man the minimum recorded is 0.006 (Helmholtz, 1866; Aubert, 1865). Taking the reciprocal of the minimum $\Delta I/I$ as a measure of maximum intensity discrimination, and putting *Drosophila* at 1, the ratios *Drosophila*/bee/man are 1/60/249 for maximum intensity discrimination, and 1/9.4/1110 for maximum visual acuity. A rough parallelism is apparent in these functions. Possibly some other, more theoretically defensible measure of maximum intensity discrimination might show a better parallelism to maximum visual acuity.

VI

Interpretation of Data

1. Visual Acuity.—*Drosophila* is the fourth organism whose visual acuity has been found to vary with illumination,—the other three being man (Koenig), the bee (Hecht and Wolf), and the fiddler crab (Clark, 1932). In each case the visual acuity is low at low intensities and increases with log I in a characteristically sigmoid manner. The only quantitative interpretation at present available for this property of visual acuity (Hecht, 1926, 1928) depends on the recognition of visual acuity as a measure of the resolving power of the retinal surface. The resolving power of a surface composed of independently functioning elements depends on the number of elements per unit area, or more specifically on the distance between the centers of the sensitive elements. To account for the required variation in number of elements at different intensities, it is assumed that the thresholds of the retinal elements vary in the retinal population much as any other characteristic of biological population. Curves have been drawn to show the threshold distribution required to account quantitatively for the data of the human eye (Hecht, 1928), and for so differently constructed an organ as the bee's eye (Hecht and Wolf, 1929). The present data with *Drosophila* (Fig. 8) show the same type of sigmoid relationship, and there is no reason to suppose that the same explanation is not available for *Drosophila*.

Fig. 10 shows the differential $\Delta v.a./\Delta \log I$. It is made from the smooth curve in Fig. 8 by finding the difference in visual acuity ($\Delta v.a.$) for points 0.2 log units apart ($\Delta \log I$) and plotting this difference against the value of log I midway between them. The resulting curve has the appearance of an ordinary, symmetrical, biological distribution. A quantitative explanation of the visual acuity data of *Drosophila* then depends on the assumption that the visual acuity is inversely proportional to the angular distance between functional ommatidia, and that the thresholds of the ommatidia along the horizontal axis of the eye are distributed according to the curve in Fig. 10.

Criticism of the ideas on which such an explanation is based has been made by Freeman (1930), by Best (1930), by Wilcox (1932), and by Wilcox and Purdy (1933). Freeman argues that since visual acuity may be varied by factors other than intensity, its variation with intensity cannot depend only on the number of elements functional. Wilcox and Purdy's criticism is an elaborate form of this standpoint. They state that our ideas are "inconsistent with the fact that acuity may vary within wide limits even though illumination remains constant." Moreover, the ideas fail "to take into account the concrete perceptual situation involved in the recognition of detail,"—which apparently means that we have offered no mechanism to explain how visual resolution takes place at all.

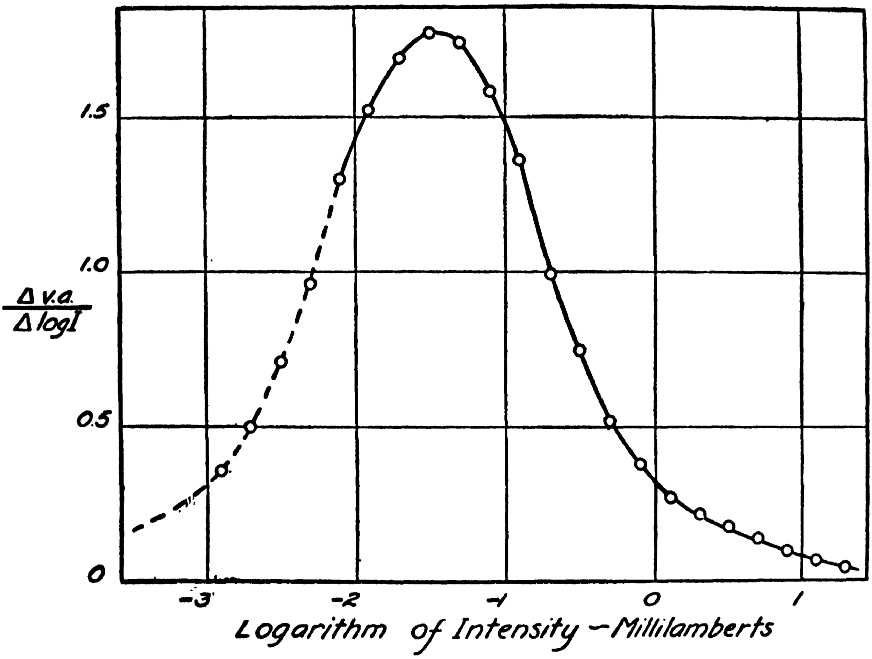

FIG. 10. Differential of the visual acuity curve of Fig. 8. The differences in visual acuity (Δ*v.a.*) between points 0.2 log units apart (Δ *log I*) in Fig. 8 are plotted against the value of the logarithm of the intensity midway between them.

It is hard to see the force of these arguments, since the idea of how visual acuity varies with intensity is quite independent of the particular mechanism which controls the magnitude of visual acuity at a given intensity. No matter what that mechanism may be, nor how complex or simple it may be, it must rest on the fact that the ultimate resolving surface is composed of units which are independent functionally. The variation of visual acuity with intensity then follows very obviously in terms of the probable distribution of thresholds. Best (1930) is worried because the necessary range of distribution of thresholds is large. However, even when the apparent range, as in the human eye, appears quite large, about 90 per cent of the variation occurs in less than 2 log units (Hecht, 1930). In the case of the bee and *Drosophila* this range is even smaller. But even if the range were very large indeed, the difficulty disappears when it is recalled that different portions of the human retina actually do possess thresholds which differ by just such large magnitudes.

Differing from these criticisms are those of Wilcox, who found that under certain conditions visual acuity does not rise steadily with log I as measured by Koenig and everyone since, but actually becomes worse at high illuminations. He then concludes that the increase in number of functional elements which presumably takes place at these high intensities cannot account for the decrease in visual acuity, and therefore the whole conception is not valid.

Wilcox used a novel procedure in his measurements. Two tiny, illuminated vertical bars each subtending 2.4 by 20 *minutes* of visual angle are viewed against an absolutely black background, and the distance is determined by which the bars must be separated for them to be recognized as two bars. It would seem almost too elementary, but apparently quite necessary, to point out that the term retinal illumination refers to the general level of illumination of the retina as a whole, or of a goodly portion of it. In Wilcox's measurements the retina as a whole is completely dark, and only the very tiny test objects are illuminated. What Wilcox measured is a glare phenomenon, and may require a new name; but it is not the relation of visual acuity to the illumination prevailing on the retina. That this criticism of his method is valid becomes clear when the reverse of this procedure is used, that is, when the test bars are black and are viewed against an evenly illuminated background. The results which Wilcox secured in this manner are in agreement with the classic data of Koenig and others, and are obviously open to the same explanation.

Wilcox himself proposes an explanation of his particular findings and in general of the relation between visual acuity and illumination, which depends on two sets of measurements made with the method already described. One set records the distance by which the two bars must be separated so that between them there appears a space which is just perceptible to the eye. The other set records the distance by which the bars must be separated so that the space between them appears of the same size as one bar. The first set Wilcox calls measurements of visual acuity; the second set, measurements of irradiation. It then appears that at different intensities the first set of measurements equals the second set of measurements multiplied by a factor. Wilcox then concludes that irradiation is the explanation of the visual acuity variation. The reverse would be equally true.

These criticisms therefore leave the original explanation of the relation between visual acuity and illumination—as due to a population distribution of thresholds of the sensitive elements—as valid as when it was proposed. This does not mean that it is the correct explanation; it is merely the only explanation which describes the data quantitatively. That is its main virtue, plus the fact that its basis is not inherently improbable, and rests on concrete assumptions with regard to the structure of the visual mechanism.

The only other explanation worth mentioning is the one given by Hoffman (Best, 1930) for the human eye. It supposes that the diffusion circles produced by two points have to be separated differently at different intensities in order to produce a recognizably lower intensity between them. This assumes that the eye can discriminate intensities absolutely—which we know it does only relatively. Even so, this explanation has never been put into quantitative form and therefore cannot be tested.

It is worth noting that the visual acuity data of *Drosophila* as given in Table II and Fig. 8 may be described with excellent precision by the stationary state equation $KI = x/(a - x)$ representing a reversible photochemical system in which the light and the dark reactions are both monomolecular. The numerical equation is $22I = (x - 2)/$

$(18 - x)$ where x equals the visual acuity multiplied by 10,000. The shape of the stationary state curve is specific with regard to the power to which x is raised in the numerator (Hecht, 1928). The human visual acuity curve (Koenig) conforms to a second power equation. The visual acuity of *Drosophila* is described by a first power equation. The visual acuity curve of the bee (Hecht and Wolf) may be described with a fair degree of approximation by a first order equation of the form $KI^2 = x/(a-x)$ in which the intensity enters as the square. The numerical form is $0.95\,I^2 = (x-3)/(160-x)$ where x is the visual acuity multiplied by 1,000. The values are direct and are not corrected for varying ommatidial angle—a correction which is useful at the very low visual acuities only.

2. *Intensity Discrimination.*—Intensity discrimination has been described theoretically (Pütter, 1918; Hecht, 1924, 1926) on the assumption that in the action of light on the photosensory system, intensities which are just recognized as different produce effects which differ by a constant increment. These increments may be recorded as changes in the frequency of discharge of the individual sensory elements, or as changes in the number of elements functional, or as both. The work on visual acuity and illumination (Hecht, 1928) favors the number idea but does not exclude frequency. The work on single end-organs (Adrian and Zotterman, 1926; Hartline and Graham, 1932) favors frequency but does not exclude number.

The quantitatively developed idea (Hecht, 1926, 1928) that the influence of intensity on visual acuity and on intensity discrimination may be described in terms of the number of elements functional has been adopted by Houstoun (Houstoun and Shearer, 1930; Houstoun, 1932) without recognizing the theoretical difficulties involved. The original supposition was that whereas visual acuity increases with the total number of active elements, each step in intensity perception corresponds only to the differential increment in the number of active elements. If the relations between visual acuity and intensity discrimination were as simple as this, the intensity at which visual acuity alters most rapidly with log I should represent the most rapid rate of entrance of functional elements, and should therefore correspond to the place where intensity discrimination is best. Moreover, at high intensities when visual acuity practically ceases to increase because nearly all the elements are already functional and very few new ones enter, intensity discrimination should be poorest—in fact almost non-existent.

Yet neither of these things is true for *Drosophila* and for the bee, and probably also for the human eye. Fig. 8 and Fig. 10 show that the maximum rate of increase in visual acuity occurs at an intensity whose logarithm is very near -1.5, whereas Figs. 3 and 6 show that at this point intensity discrimination is by no means maximal. Moreover at the highest intensities, when visual acuity has reached its top value, intensity discrimination instead of being at its worst is actually at its best. Exactly the same is true for the bee, where the maximum rate of increase in visual acuity comes very nearly at $\log I = 0$ (Hecht and Wolf) at a point where intensity discrimination is certainly not at its best (Wolf, 1933). Moreover when visual acuity has reached its maximum at $\log I = 2.0$, and intensity discrimination should be worst, it is nevertheless also at its best in the bee and shows no sign of falling off at the highest intensities.

These failures in the correspondence of the two functions are very important, and cannot be due to any chance shift in the intensities for the two functions. We are certain that for *Drosophila* the intensities for visual acuity and intensity discrimination are exactly comparable, because both functions for many of the animals were measured within a very short time of each other with the same piece of apparatus. Similarly for the bee, the two functions were measured with practically identical apparatus using the identical striped plates, and the measurements were made by the same person (Wolf). But even if the intensities were not exactly comparable, the fact that at the highest intensities both functions are maximal is adequate evidence against the interpretation.

Thus in *Drosophila* and in the bee, intensity discrimination does not depend on the rate at which elements become functional, but apparently rather on the total number of elements functional, as in visual acuity. It is possible that though both are functions of the total number of elements active in a given unit of sensory surface, the specific relation is different for each. For visual acuity the situation is simple, but for intensity discrimination it means a revision of ideas held up to now. One possibility is that intensity discrimination is similar and constant for all the sensory elements, but that the thresholds have a probability distribution. Then increasing the total number of elements decreases the sensory contribution each has to make to produce a constant increment in total sensory effect, and therefore the fraction $\Delta I/I$ becomes smaller as the total number of elements increases. Other possibilities are also available, but a discussion of them is unfruitful at the present stage of our knowledge.

SUMMARY

Drosophila possesses an inherited reflex response to a moving visual pattern which can be used to measure its capacity for intensity discrimination and its visual acuity at different illuminations. It is found that these two properties of vision run approximately parallel courses as functions of the prevailing intensity.

Visual acuity varies with the logarithm of the intensity in much the same sigmoid way as in man, the bee, and the fiddler crab. The resolving power is very poor at low illuminations and increases at high illuminations. The maximum visual acuity is 0.0018, which is 1/1000 of the maximum of the human eye and 1/10 that of the bee.

The intensity discrimination of *Drosophila* is also extremely poor, even at its best. At low illuminations for two intensities to be recognized as different, the higher must be nearly 100 times the lower. This ratio decreases as the intensity increases, and reaches a minimum of 2.5 which is maintained at the highest intensities. The minimum value of $\Delta I/I$ for *Drosophila* is 1.5, which is to be compared with 0.25 for the bee and 0.006 for man.

An explanation of the variation of visual acuity with illumination is given in terms of the variation in number of elements functional in the retinal mosaic at different intensities, this being dependent on the general statistical distribution of thresholds in the ommatidial population. Visual acuity is thus determined by the integral form of this distribution and corresponds to the total number of elements functional. The idea that intensity discrimination is determined by the differential form of this distribution—that is, that it depends on the rate of entrance of functional elements with intensity—is shown to be untenable in the light of the correspondence of the two visual functions. It is suggested that, like visual acuity, intensity discrimina-

tion may also have to be considered as a function of the total number of elements active at a given intensity.

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ON GUAIACOL SOLUTIONS

I. THE ELECTRICAL CONDUCTIVITY OF SODIUM AND POTASSIUM GUAIACOLATES IN GUAIACOL

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INTRODUCTION

Chemists need information about the behavior of electrolytes in non-aqueous solutions. Biologists likewise need this information since there is considerable evidence that the protoplasmic surface consists of such substances and they have been employed in models designed to imitate the action of the living cell.

A quantitative relationship between the equivalent electrical conductance and the concentration of dilute electrolytic solutions in which the solute is considered to be entirely in the form of free ions has been derived theoretically by Onsager,[1] following the work of Debye and Hückel.[2] In this treatment the decrease in equivalent conductance with increasing concentration is attributed to a decrease in the ionic mobilities arising from the influence of interionic electrostatic forces. For strong electrolytes in water, calculations based on Onsager's equations are in good agreement with measurements on dilute solutions.[2,3]

However, similar calculations, based on the assumption of complete ionization, do not adequately represent the facts for electrolytes in most non-aqueous solvents which, in general, have dielectric constants appreciably lower than that of water. In such solvents all electrolytes appear to be "weak," and measurements on these solutions seem to indicate that a considerable portion of the solute is not in the form of "free" ions.

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[1] Onsager, L., *Phys. Z.*, 1927, **28**, 277.

[2] Debye, P., and Hückel, E., *Phys. Z.*, 1923, **24**, 305.

[3] Shedlovsky, T., *J. Am. Chem. Soc.*, 1932, **54**, 1411; MacInnes, D. A., Shedlovsky, T., and Longsworth, L. G., *Chem. Rev.*, 1933, **13**, 29.

It will be shown in this paper that the electrical conductivity of solutions of sodium and potassium guaiacolates in guaiacol can be accounted for satisfactorily by assuming an ionic equilibrium in addition to the interionic attraction effects on the mobilities of the ions.

Mass action constants for sodium guaiacolate and potassium guaiacolate can thus be calculated from conductivity measurements. It is desirable, however, to obtain these constants if possible from another type of measurement. Such an independent method is available in the determination of the partition coefficients of the electrolyte between guaiacol and water,[4] the two liquids forming non-miscible phases. Since, however, the nature of distribution experiments is such as to render the two non-miscible liquids saturated with each other, a fair comparison between the two methods for determining the ionization constants can be made only if the conductivity measurements also refer to solutions in guaiacol saturated with water. Consequently, the conductivity measurements reported in this paper, from which the ionization constants are computed, were made on solutions containing guaiacol from the same source and with practically the same water content as in the case of the subsequent distribution experiments.

In computing the results from the conductivity data it is necessary to know the dielectric constant, viscosity, and density of the "wet" guaiacol solvent. These values were measured and are reported in this paper, since they are not available in the literature. For completeness the corresponding values for dry guaiacol are also given. The same computations which give the ionization constants of the alkali guaiacolates in wet guaiacol from conductivity determinations, also give the limiting equivalent conductance values (Λ_0), corresponding to the sum of the positive and negative ion mobilities at zero concentration. These (Λ_0) values have been combined with corresponding transference numbers, yielding values for the limiting mobilities of potassium, sodium, and guaiacolate ions in wet guaiacol.

THEORETICAL

In discussing weak electrolytic solutions we shall assume that only a portion of the solute is in the form of free ions able to carry current, while the rest can be considered "undissociated," and that a corresponding ionic equilibrium exists in accordance with a mass action equation. However, it is not necessary to assume that the undissociated solute consists of stable molecules bound by chemical or quantized forces. We prefer to consider them to be ion pairs as suggested by Bjerrum.[5]

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[4] The distribution of sodium and potassium guaiacolate between water and guaiacol is the subject of the following paper.

The electrostatic force between a pair of ions of opposite charge which have approached to "contact" tends to hold them together as a stable pair. The mutual potential energy of this ion pair, in accordance with Coulomb's law, depends on the charges, the dielectric constant of the medium, and the distance between the charge centers (the sum of the ionic radii, if we consider the ions to be spherical). Now, if the average kinetic energy of the solvent molecules is greater than this potential energy, the ion pair cannot exist for any appreciable time. This is the case for strong uni-univalent electrolytes in water. However, if the average kinetic energy of the solvent molecules is less than the mutual potential energy of the ion pair, it will exist until it is struck by a solvent molecule having sufficient kinetic energy to break it up.

This viewpoint predicts that there should be relatively more ion pairs (undissociated solute) when the dielectric constant is low, when the ions are small, when the charges are large, and when the temperature is low. Calculations of ionization constants as a function of ion size, dielectric constant, etc., based on this view were made by Bjerrum[5] for electrolytes in solvents of relatively high dielectric constant, and more recently by Fuoss and Krauss[6] for tetraisoamylammonium nitrate in dioxane-water mixtures, and for silver nitrate in benzonitrile, acetonitrile, and ammonia.

Our problem is to interpret the electrical conductivity of weak electrolytic solutions; namely, of sodium guaiacolate and potassium guaiacolate in guaiacol. The mass action equation for the ionic equilibrium we have assumed is

$$K = \frac{(A_{M^+})\,(A_{G^-})}{(A_{M^+G^-})} = \frac{(C\,\theta\gamma)^2}{C(1-\theta)} \tag{1}$$

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[5] Bjerrum, N., *K. Danske Vidensk. Selsk.*, 1926, **7**, No. 9.

[6] Fuoss, R. M., and Kraus, C. A., *J. Am. Chem. Soc.*, 1933, **55**, 1019.

in which A_{M^+} is the activity of the positive (metal) ions, A_{G^-} is the activity of the negative (guaiacolate) ions, and $A_{M^+G^-}$ is the activity of the undissociated ions, or paired ions. C is the concentration, θ the "degree of dissociation," and γ the mean ionic activity coefficient. We assume that the undissociated portion behaves as a normal solute, and consequently has an activity equal to its concentration.

The mean ionic activity coefficient will be computed from the Debye-Hückel equation

$$-\log_{10}\gamma = \frac{a\sqrt{C\theta}}{1 + b\sqrt{C\theta}} \tag{2}$$

in which

$$a = \frac{0.4343\, e^3}{(DkT)^{3/2}} \sqrt{\frac{2\,\pi N}{1000}}$$

and

$$b = \sqrt{\frac{8\,\pi N e^2 r^2}{1000\, DkT}};$$

e = electronic charge, N = Avogadro's number, k = Boltzmann's constant, D = dielectric constant, T = absolute temperature, r = distance of closest approach between ions. For water-saturated guaiacol at 25°C. $a = 6.52$, and for sodium and potassium guaiacolates in this solvent we have used an approximate value of 7 Å. for r (estimated from crystal structure data), which gives a value of $b = 5.36$. Actually, of course, r is not the same for the two salts, but nearly enough so for the present purpose, since the term $b\sqrt{C\theta}$ enters as a correction. We shall assume that the Onsager equation adequately represents the relationship between the equivalent conductance and the concentration for the dissociated (free) ions. For a completely dissociated electrolyte this is $\Lambda_\epsilon = \Lambda_0 - A\sqrt{C_i}$, in which Λ_ϵ is the equivalent conductance, Λ_0 the limiting value of equivalent conductance at zero concentration, C_i is the ionic concentration,

$$A = \alpha\Lambda_0 + \beta = \frac{8.18 \times 10^{-5}}{(DT)^{3/2}}\Lambda_0 + \frac{82}{\eta\,(DT)^{1/2}},$$

η being the viscosity, and D and T having the same meaning as above. For water-saturated guaiacol $A = 2.93\,\Lambda_0 + 19.36$. The equivalent conductance, Λ, is defined as $\Lambda = \frac{100\,\overline{L}}{C}$, $\overline{L}$ being the specific conductance; thus $\Lambda_\epsilon = \frac{1000\,\overline{L}}{C_i}$.

Since $C_i = C\theta$, the Onsager equation gives us

$$\Lambda/\theta = \Lambda_0 - (\alpha\,\Lambda_0 + \beta)\sqrt{C\theta}$$

or

$$\theta = \frac{\Lambda}{\Lambda_0 - (\alpha\,\Lambda_0 + \beta)\sqrt{C\theta}} \tag{3}$$

Fuoss and Kraus[7] have discussed the mathematical treatment of these equations and have indicated a convenient method of solution. It consists of assuming a provisional value of Λ_0 and then computing values of θ for the series of measurements, from equation (3) which is conveniently solved by successive approximations. Corresponding values of γ are then obtained from equation (2), and values of $\gamma\sqrt{C}$ are plotted against $\frac{\sqrt{1-\theta}}{\theta}$. Since equation (1) can be easily transformed to $\frac{\sqrt{K}\sqrt{1-\theta}}{\theta}\,\gamma\sqrt{C}$, such a plot should give a straight line passing through the origin, with a slope of $\sqrt{K}$, if the correct value of Λ_0 has been chosen. Successive values of Λ_0 are assumed until this is found to be the case.

With the values of Λ_0 and of K thus determined from a series of conductance measurements, a theoretical conductance curve corresponding to these constants can be computed by reversing the calculations described above. A direct graphical comparison between the measured and computed conductance values is thus afforded.

EXPERIMENTAL

1. Guaiacol.—Synthetic guaiacol (Kahlbaum's C.P. crystalline guaiacol) was redistilled under reduced pressure in a slow stream of nitrogen, using an all glass (Pyrex) distilling apparatus with a fractionating column. The distillate was protected from atmospheric moisture. Colorless fractions, having melting points from 28.0—28.36°C., were collected.[8]

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[7] Fuoss, R. M., and Kraus, C. A., *J. Am. Chem. Soc.*, 1933, **55**, 476.

[8] The freezing point of guaiacol is quite sensitive to traces of impurities because of its high cryoscopic constant. We determined the molal freezing point lowering (ΔT/mole of solute in 1 liter of solution) with water, toluene, and carbon tetrachloride. The values were 5.4°, 6.1°, and 6.4° respectively.

Although the melting point of guaiacol is several degrees above the temperature at which our conductivity measurements were made, namely 25°C., no difficulties from crystallization were encountered due to the readiness with which this substance supercools.

The solubility of water in guaiacol was determined from density measurements on a series of solutions containing known quantities of water in the solvent guaiacol. A plot of density against water content, extrapolated to the density of guaiacol saturated with water, was used for this purpose. The density measurements were made with a Sprengel pyknometer. Physical properties of dry and water-saturated guaiacol at 25°C. are listed in Table I.

TABLE I

Physical Properties of Dry and Water-Saturated Guaiacol at 25°C.

	Anhydrous guaiacol	Water-saturated guaiacol
Density	1.1289	1.1275
Dielectric constant*	11.8	14.3
Relative viscosity†	6.10	7.23
Specific conductance	1×10^{-8}	$3\text{–}5 \times 10^{-8}$
Solubility of water in guaiacol = 4.60 gm. water/100 gm. solution		

* We are indebted to Dr. S. O. Morgan of the Bell Telephone Laboratories for the dielectric constant measurements.

† The viscosities (relative to water at 25°C.) were measured in an Ostwald viscosimeter. The relative viscosity values for various water contents in the guaiacol were

Per cent water	0	0.9	2.3	4.6 (saturated)
Relative viscosity	6.10	6.44	6.81	7.23

2. Sodium and Potassium Guaiacolates.—The alkali guaiacolates were prepared by the reaction of the ethylates with guaiacol in a medium of anhydrous ethyl alcohol, from which the salts were subsequently crystallized. Freshly made alkali metal wire was introduced directly into anhydrous alcohol through a sodium press. After the metal had dissolved, an equivalent quantity of guaiacol was introduced. The crystallized salt was washed several times with anhydrous ether and then dried in an Abderhalden (vacuum) drier. The purity of the product was checked by titrating weighed samples with standard hydrochloric acid, and computing the alkali metal content.

The solubilities of the salts in anhydrous and water-saturated guaiacol at 25°C. were determined also by titration with standard acid. The results, expressed in moles of salt per thousand grams of solvent, were:

	Anhydrous guaiacol	Water-saturated guaiacol
Potassium guaiacolate	0.0071	0.0763
Sodium guaiacolate	0.0063	0.154

3. Conductivity Measurements.—The conductivity measurements were made in a "flask cell" of the type described by one of us,[9] having a cell constant of 0.0832. An alternating current conductivity bridge and an oil thermostat at 25.000°C. were used.[10] A known quantity of guaiacol (about 500 cc.) which had been almost, but not quite, saturated with conductivity water[11] was introduced into the cell and its conductance was measured. Then, successive weighed increments of a relatively concentrated stock solution of known guaiacolate content were added, and corresponding conductance determinations were made. Thus it was possible to measure the conductance of a whole series of concentrations in the same solvent. The solution in the cell was protected from atmospheric impurities at all times with purified nitrogen.[9]

It was interesting to know the effect of the water content of the solvent guaiacol on the conductance. This was determined by making a series of measurements, starting with a solution of guaiacolate in anhydrous guaiacol and successively increasing the water content.

RESULTS

The results of the conductivity measurements on solutions of sodium guaiacolate and potassium guaiacolate in wet guaiacol are summarized in Tables II and III respectively. The listed values of equivalent conductance have not had a solvent correction applied to them. We have assumed that the conductance of the solvent is due to ions arising from guaiacol, and this dissociation is practically repressed by the very much greater number of guaiacolate ions supplied by the salts.

The fact that the molal freezing point lowering of guaiacol with water was found to be no greater than with toluene or carbon tetrachloride, is definite evidence against the possibility of ions arising from the water in the solvent. For these reasons, and because the solutions were protected against foreign impurities throughout the course of the measurements, we believe that the solvent corrections should be neglected.

The effect of water content on the conductivity of sodium guaiacolate in guaiacol (C = 0.0009 molal) is shown in Fig. 1, in which the ratio of the equivalent conductance (Λ) to the equivalent conductance in anhydrous guaiacol (λ°) is plotted against the water content, expressed as per cent of (water) saturation. The curve rises from a value of 1 at zero water content to 34.5 at 100 per cent (water) saturation.

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[9] Shedlovsky, T., *J. Am. Chem. Soc.*, 1932, **54**, 1415.

[10] Shedlovsky, T., *J. Am. Chem. Soc.*, 1930, **52**, 1793.

[11] This was done to make sure that no water would separate out in the subsequent additions of solute. The dielectric constant and viscosity of the nearly saturated guaiacol can be safely assumed to be the same as that of the completely saturated solvent for our purposes.

TABLE II

Conductance of Sodium Guaiacolate in Wet Guaiacol at 25°C.
Water content = 99 per cent saturated
Specific conductivity = 5.18×10^{-8}

Concentration mole/liter	$\Lambda = \frac{\text{Specific conductivity}}{\text{Concentration}} \times 10^3$	
	Observed	Calculated
0	—	7.50
0.000206	2.893	2.835
0.000771	1.707	1.717
0.00224	1.097	1.100
0.00454	0.809	0.813
0.00901	0.601	0.602
0.01666	0.460	0.461

TABLE III

Conductance of Potassium Guaiacolate in Wet Guaiacol at 25°C.
Water content = 98 per cent saturated
Specific conductivity = 2.84×10^{-8}

Concentration mole/liter	$\Lambda = \frac{\text{Specific conductivity}}{\text{Concentration}} \times 10^3$	
	Observed	Calculated
0	—	8.00
0.000442	2.461	2.463
0.000959	1.808	1.819
0.002151	1.300	1.301
0.003719	1.037	1.032
0.005765	0.862	0.857

The relatively great increase in conductance (over thirtyfold), cannot be satisfactorily explained on the basis of difference in the dielectric constants of anhydrous guaiacol ($D = 11.8$) and wet guaiacol ($D = 14.3$). It is rather due to the fact that the water molecules (whose mole fraction is about $\frac{1}{4}$ in water-saturated guaiacol) are definitely more polar than guaiacol molecules, and tend to orient themselves with some stability around the salt ions in the solution. This polarization of water dipoles in the strong electric fields around the ions may be regarded as an hydration which has the effect of increasing the size of the ions.

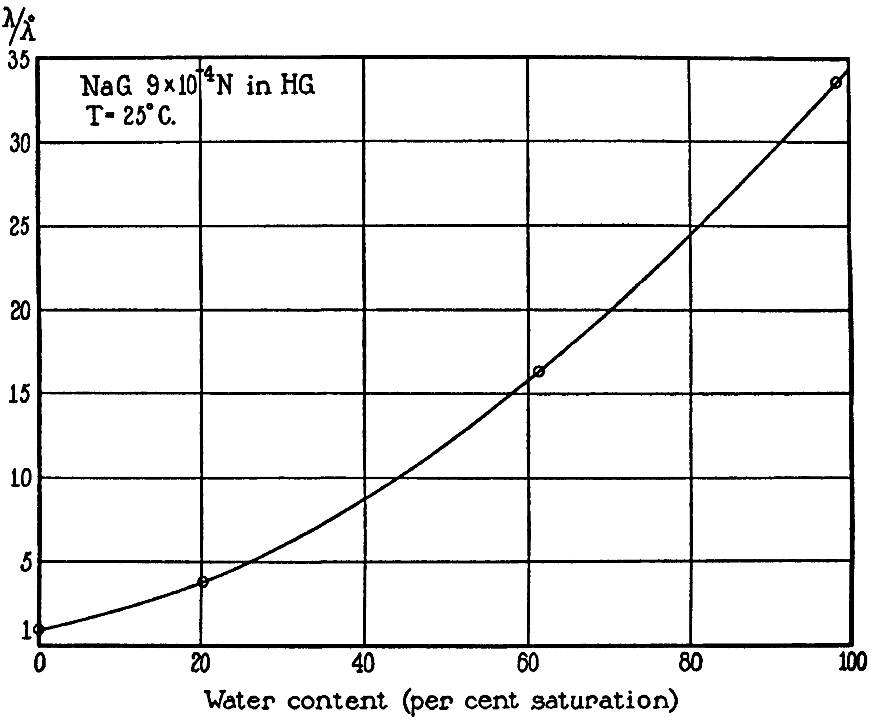

FIG. 1. Effect of water content on the conductance of sodium guaiacolate in guaiacol, expressed as the ratio of the observed conductance (λ) to the conductance in anhydrous guaiacol (λ°).

It has been pointed out in the theoretical portion of this paper that, other things being equal, an increase in the size of ions tends to increase the "strength" of an electrolyte. On this basis, we may attribute the conductance increase with increasing water content to a greater number of free ions, capable of carrying current as the average size of the ions is increased.

From the results shown in Tables II and III values of the dissociation constants and the limiting conductances of sodium and potassium guaiacolates were computed in the manner previously discussed, using the equations

$$K = \frac{C\theta\gamma^2}{1-\theta} \tag{1}$$

$$-\log_{10}\gamma = \frac{6.52\sqrt{C\theta}}{1 + 5.36\sqrt{C\theta}} \tag{2}$$

$$\theta = \frac{\Lambda}{\Lambda_0 - (2.93\,\Lambda_0 + 19.36)\sqrt{C\theta}} \tag{3}$$

The results of the computations are tabulated in Tables IV and V.

TABLE IV

Sodium Guaiacolate. $\Lambda_0 = 7.50$

C (mole/liter)	θ (degree of dissociation)	γ (mean ionic activity coefficient)	$K \times 10^5$
0.000206	0.406	0.877	4.38
0.000771	0.2463	0.825	4.22
0.00224	0.1635	0.771	4.25
0.00454	0.1241	0.727	4.22
0.00901	0.0956	0.683	4.25
0.01666	0.0764	0.638	4.28
			Average 4.27×10^{-5}

TABLE V

Potassium Guaiacolate. $\Lambda_0 = 8.00$

C (mole/liter)	θ (degree of dissociation)	γ (mean ionic activity coefficient)	$K \times 10^5$
0.000442	0.3288	0.844	5.07
0.000959	0.2462	0.808	5.02
0.002151	0.1818	0.765	5.07
0.003719	0.1484	0.731	5.13
0.005765	0.1258	0.701	5.13
			Average 5.08×10^{-5}

In Fig. 2 are shown plots of $\frac{\sqrt{1-\theta}}{\theta}$ against $\gamma\sqrt{C}$. From equation (1) we obtain $\frac{\sqrt{1-\theta}}{\theta} = \frac{1}{\sqrt{K}}\gamma\sqrt{C}$, so that if the correct values of

Λ_0 have been chosen, these plots should be straight lines, passing through the origin, as is the case. The slope of these lines is $1/\sqrt{K}$.

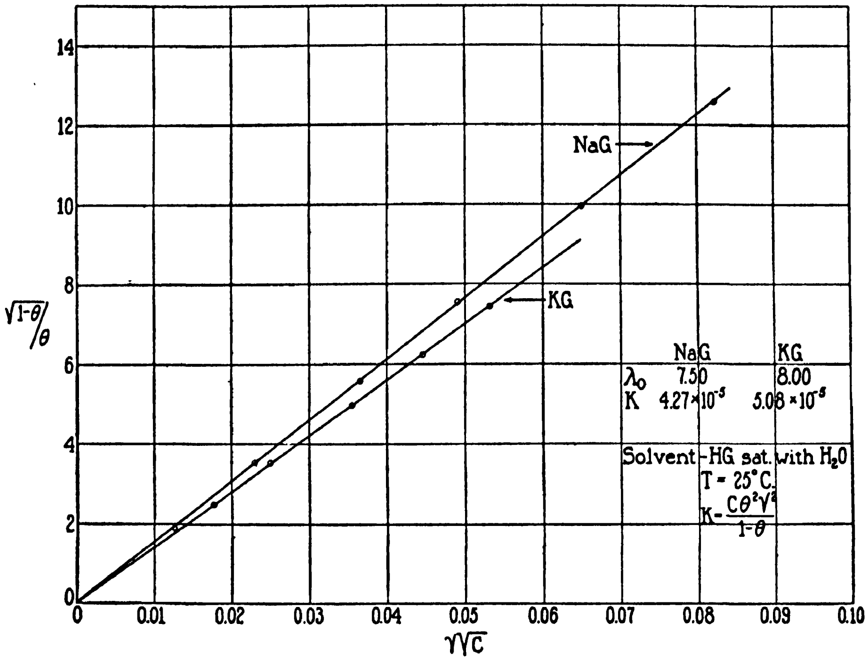

FIG. 2. Plots of $\sqrt{\frac{1-\theta}{\theta}}$ against $\gamma\sqrt{C}$ for sodium and potassium guaiacolates in water-saturated guaiacol.

TABLE VI

Solvent: Guaiacol Saturated with Water. T = 25°C.

	Λ_0	T_+	$l_0(G^-)$	$l_0(+)$
Sodium guaiacolate....................	7.50	0.54	3.45	4.05 (Na^+)
Potassium guaiacolate..................	8.00	0.57	3.44*	4.56 (K^+)

* The viscosity of wet guaiacol, being 7.2 times that of water, we can estimate the magnitude of the limiting conductance of the guaiacolate ion to be about 25 in water, assuming the validity of Stokes' law; that is, $l_0(G^-)$ in water = 7.2 (3.45) = 25. This is an entirely reasonable value. However, a similar computation gives 29 for the sodium ion and 33 for the potassium ion, whereas these values should be about 50 and 76 respectively. The discrepancy is probably due to the fact that, although Stokes' law seems to hold for large ions, such as the guaiacolate ion (6 Å.), it fails for the smaller ions.

We are indebted to Dr. L. G. Longsworth of The Rockefeller Institute for supplying us with values for the transference numbers (T_+) for

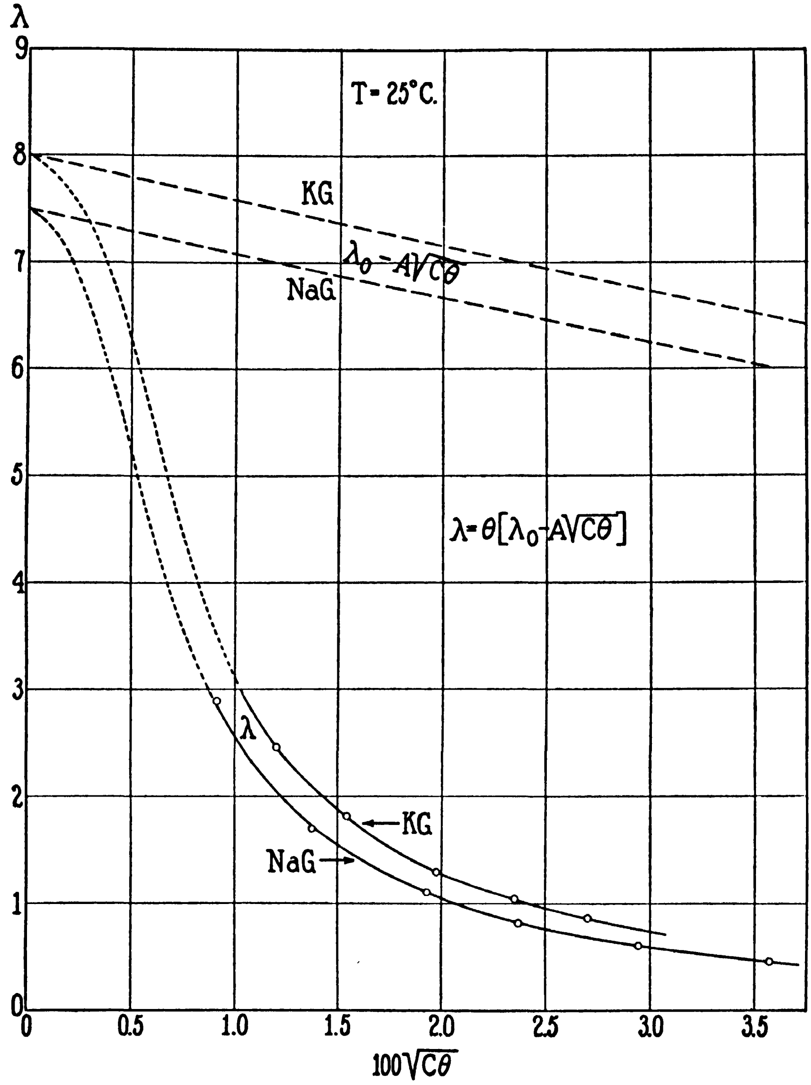

FIG. 3. The conductance of sodium and potassium guaiacolates in water-saturated guaiacol.

sodium and potassium guaiacolates in wet guaiacol, obtained by the moving boundary method. By combining them with our limiting conductance values (Λ_0) we are able to obtain the limiting conductances (l_0) of sodium, potassium, and guaiacolate ions. In Table VI these quantities are listed.

Using the values $\Lambda_0 = 7.50$, $K = 4.27 \times 10^{-5}$ for sodium guaiacolate, and $\Lambda_0 = 8.00$, $K = 5.08 \times 10^{-5}$ for potassium guaiacolate, calculated values for the equivalent conductance, Λ, of these salts at the concentrations measured were obtained. These are shown in the last column of Tables II and III, and agree reasonably well with the values actually observed.

We may therefore conclude that the conductivity of the alkali guaiacolates in water-saturated guaiacol can be satisfactorily accounted for on a basis of an ionic equilibrium, assuming that the "associated" portion of the salt behaves as an "ideal" solute, and correcting for the effects of interionic attractions on the mobilities and activities of the free ions present. From this point of view these salts were found to be weak electrolytes, not unlike acetic acid in water.

SUMMARY

1. Measurements on the densities, viscosities, dielectric constants, and specific conductances of pure anhydrous and water-saturated guaiacol at 25°C. are reported.

2. The solubility of water in guaiacol at 25°C., and its effect on the electrical conductivity of a sodium guaiacolate solution is given.

3. Electrical conductivity measurements are reported on solutions of sodium and potassium guaiacolates in water-saturated guaiacol at 25°C.

4. The decrease of electrical conductivity with increasing concentration for these salts is explained on the basis of an ionic equilibrium combined with the interionic attraction theory of Debye and Hückel.

5. The limiting equivalent conductances of sodium and potassium guaiacolates in water-saturated guaiacol at 25°C., the corresponding limiting ionic mobilities, and the dissociation constants are computed from the conductivity measurements. The salts are found to be weak electrolytes with dissociation constants of the order of 5×10^{-5}.

ON GUAIACOL SOLUTIONS

II. THE DISTRIBUTION OF SODIUM AND POTASSIUM GUAIACOLATES BETWEEN GUAIACOL AND WATER

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INTRODUCTION

In a series of studies related to the kinetics of penetration in living cells Osterhout and his coworkers[1] have used artificial cell models, consisting of a non-aqueous phase (guaiacol or guaiacol with *p*-cresol), representing the protoplasmic surface, in contact with aqueous phases, representing the cell sap and the external solution. In this connection the distribution of electrolytes between guaiacol and water, is particularly interesting.

The electrical conductivities of sodium and potassium guaiacolate solutions in guaiacol have been discussed in the previous paper.[2] It was shown that these salts are weak electrolytes in such solutions, and that their ionization constants can be computed from the conductivity measurements by taking into account the effect of interionic forces on the mobilities and activities of the ions.

This paper will deal with the partition of these salts between water and guaiacol, and will provide an independent means for computing these ionization constants. Starting from the assumptions that the salts are weak electrolytes in the guaiacol phase and strong electrolytes in the aqueous phase, and using the interionic attraction theory of Debye and Hückel, it is shown that the variation of the distribution coefficients with concentration can be explained. Furthermore, the ionization constants previously obtained from conductivity measurements are found to be in satisfactory agreement with the distribution determinations.

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[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 157, 529. Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931–32, **15**, 667. Osterhout, W. J. V., Kamerling, S. E., and Stanley, W. M., *J. Gen. Physiol.*, 1933–34, **17**, 445, 469.

[2] Shedlovsky, T., and Uhlig, H. H., *J. Gen. Physiol.*, 1933–34, **17**, 549.

Also, it is shown that the magnitude of the distribution coefficients can be predicted from theoretical considerations, using only the properties of the solvent and the solute in the computations.

THEORETICAL

In discussing the distribution of sodium or potassium guaiacolate between guaiacol and water, we shall assume that the salt is practically completely dissociated in the aqueous phase, in which, therefore, the ionic activity is $(C_1\gamma_1)$, – (C_1) being the total concentration of the salt in this phase, and (γ_1) the corresponding activity coefficient.

In the non-aqueous phase, the salt is only partially dissociated in accordance with the ionic equilibrium $M^+ + G^- = MG$ (M referring to the metal and G to guaiacolate), for which the mass action expression is

$$K = \frac{[M^+]\,[G^-]}{[MG]} = \frac{(C\theta\gamma)^2}{C(1 - \theta)} \tag{1}$$

In this equation, K is the ionization constant, the brackets refer to activities, C is the total concentration in the non-aqueous phase, θ is the degree of ionization, and γ is the mean ionic activity coefficient. We have assumed that the undissociated portion of the electrolyte behaves as an "ideal" solute, so that its activity is equal to its concentration. At equilibrium, the ratio of the activities of any solute species distributed between two phases, at a fixed temperature and pressure, is a constant,[3] independent of the concentration. Accordingly, for the dissociated portion of the solute

$$S_0 = \frac{C\theta\gamma}{C_1\,\gamma_1} \tag{2}$$

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[3] In general this constant is not unity. This is because two standard states are involved, one for each phase. The actual "escaping tendency" of a solute species in equilibrium between two phases must, however, be equal. In other words, if the activities in the two phases were referred to a single standard state (in the gaseous or crystal state, for example), then the limiting partition coefficient would be unity. It is customary, however, to define the activity coefficient in such a manner as to render it equal to unity at infinite dilution, since by so doing the activity approaches the concentration as the solution becomes infinitely dilute. This procedure involves the fixing of a separate standard state for each solvent.

Also, it is assumed that the properties of the solvents remain essentially constant.

in which the numerator on the right hand side of the equation is the ionic activity in the non-aqueous phase, and the denominator is the corresponding activity in the aqueous phase. The value of (S_0), the limiting partition coefficient, depends on the standard states for the two solvents.

We shall assume that the activity coefficients (γ) and (γ_1) are given by the familiar Debye-Hückel equations

$$\log_{10} \gamma = -\frac{a\sqrt{C\theta}}{1 + b\sqrt{C\theta}} \text{ for the non-aqueous phase} \tag{3}$$

and

$$\log_{10} \gamma_1 = -\frac{a_1\sqrt{C_1}}{1 + b_1\sqrt{C_1}} \text{ for the aqueous phase} \tag{3'}$$

in which the coefficients in the numerators are equal to

$$\frac{0.4343\, \epsilon^2}{2DkT}\left(\frac{8\pi N\epsilon^2}{1000\, DkT}\right)^{\frac{1}{2}}$$

and the coefficients in the denominators are equal to

$$\left(\frac{8\pi N\epsilon^2}{1000\, DkT}\right)^{\frac{1}{2}} d$$

where

ϵ = electronic charge = 4.77 × 10^{-10} e.s.u.
k = Boltzmann's constant = 1.371 × 10^{-16} erg/degree
D = dielectric constant
T = absolute temperature
N = Avogadro's number = 6.06 × 10^{23}
d = distance of closest approach for the ions

Combining (1) and (2), and substituting $\frac{C}{C_1} = S$, the partition coefficient, we obtain

$$S = \frac{(S_0)^2}{K} \cdot \frac{C_1(\gamma_1)^2}{(1-\theta)} = \frac{\beta}{1-\theta} C_1(\gamma_1)^2 \tag{4}$$

in which $\frac{S_0^2}{K} = \beta$. Taking logarithms in (1) and substituting from (3) and (3′)

$$\log \theta = \log S_0 - \log S + \frac{a\sqrt{C\theta}}{1 + b\sqrt{C\theta}} - \frac{a_1\sqrt{C_1}}{1 + b_1\sqrt{C_1}} \tag{5}$$

The total salt concentrations, C and C_1, in the non-aqueous and aqueous phases respectively, have been determined by a method which will be described later in this paper. The ratio of these concentrations, S, is therefore also known, and γ_1 can be computed from equation (3′). Assuming a provisional value for S_0, which may be obtained from a plot of S *vs.* $C_1\gamma_1^2$, extrapolated to $C_1\gamma_1^2 = 0$, values of θ corresponding to the various distribution experiments, have been computed from equation (5) by a method of successive approximations. Then, using equation (4), values of $S(1 - \theta)$ have been plotted against the corresponding values of $C_1\gamma_1^2$. If the correct value of S_0 has been chosen, the plot will be linear, passing through the origin with a slope equal to β (see equation (4)). Successive values of S_0 are assumed until this criterion is satisfied. Since the slope of the plot is $\beta = \frac{(S_0)^2}{K}$, the ionization constant, K, can thus be evaluated.

In this discussion we have neglected the possible effect of hydrolysis of the salts in the aqueous phase on the partition coefficients. Although guaiacol is such a weak acid that its salts are extensively hydrolyzed in pure water, it will be shown below that the solubility of guaiacol in the aqueous phase, which, of course, was always saturated with guaiacol in the partition experiments, is sufficiently great to reduce hydrolysis to a negligible extent for our purpose.

Effect of Hydrolysis on the Partition Coefficients

The hydrolysis of sodium and potassium guaiacolate: $M^+ + G^- + H_2O = M^+ + OH^- + HG$ has a mass action constant

$$K_h = \frac{[OH^-]\,[HG]}{[G^-]} = \frac{K_w}{K_{HG}}. \tag{6}$$

K_w, in this expression, is the dissociation constant for water (1×10^{-14} at 25°) and K_{HG} is the corresponding dissociation constant of guaiacol in water.

Since we are interested only in the order of magnitude of the hydrolysis, we may neglect activity corrections. Thus, if C_1 is the original concentration of guaiacolate in the aqueous phase, (X) the concentration of salt hydrolyzed, and C_0 the solubility of guaiacol in water, then

$$K_h = \frac{K_w}{K_{HG}} = \frac{(C_0)X}{(C - X)} \qquad (6')$$

The solubility of guaiacol in water (C_0) was determined by making successive additions of guaiacol from a weight burette to a weighed quantity of water contained in a glass-stoppered flask. After each addition of guaiacol, the solution was thoroughly shaken and allowed to stand in a constant temperature room at 25°C. The amount of guaiacol added to 116 gm. of water necessary for saturation was determined to within 1 drop.

Solubility (weights in air) gm. HG/1000 gm. H_2O = 24.1 ± 0.2

Moles guaiacol per 1000 cc. solution = 0.190 = C_0

The dissociation constant of guaiacol K_{HG} in water at 25°C. was determined in the following way. The pH of an aqueous solution 0.129 molal in guaiacol and 0.0195 molal in potassium guaiacolate was measured with the glass electrode, giving a value of 9.20.[4]

Taking logarithms in equation (6) we obtain $\log K_{HG} = \log [G^-] - \log [HG] - \text{pH}$, from which the value of the dissociation constant K_{HG} can be calculated by substituting the appropriate values for $[G^-]$, pH, and $[HG]$; $[HG] = 0.129$, pH = 9.20, and $\log [G^-] = \log (0.0195) + \log \gamma_{G^-}$. The activity coefficient term, $\log \gamma_{G^-}$ is given by the Debye-Hückel equation [equation (3′)]. Thus, K_{HG} at 25°C. was found to be 8×10^{-11}.

Returning now to equation (6′), and substituting the proper values for K_w, K_{HG}, and C_0 we obtain:

$$K_h = \frac{10^{-14}}{8 \times 10^{-11}} = \frac{0.190\,X}{C - X}$$

Solving for X, we get

$$X = (6.6 \times 10^{-4})C$$

which shows that the maximum error to be expected from neglecting hydrolysis is under a tenth of a per cent. For our purposes this small effect is entirely negligible.

Partition Experiments

The partition experiments were carried out in Pyrex glass tubes of about 50 cc. capacity, provided with a stop-cock at one end and a ground glass stopper at the other. With this arrangement, the two liquid phases could readily be separated from each other for subsequent analysis. Equilibrium between the aqueous and guaiacol solutions of the alkali guaiacolates[5] of various concentrations was attained by rotating the tubes containing the solutions in a constant temperature room at 25°C. ± 0.1° for 15 hours. The solutions turned from colorless to a yellow or brownish tint at the end of this period, due probably to an oxidation of a small amount of guaiacol to a quinoid structure.

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[4] We are indebted to Mr. D. Belcher for this measurement. Pure materials, and carbon dioxide-free conductivity water were used in preparing the solution.

Analysis.—The analysis of both the aqueous and guaiacol phases for sodium or potassium guaiacolate was carried out by adding distilled water to a known volume of solution and titrating with standard hydrochloric acid. Analyses of sufficient accuracy were only made possible by employing with glass electrodes a modification of the differential titration method described by MacInnes and Dole.[6] Glass electrodes were used since they are not "poisoned" by guaiacol. The procedure was to titrate the major portion of the solution with 0.1 N or 0.01 N acid delivered from a weight burette to within a few drops of the end-point, and then to complete the titration with a more dilute standard acid (0.001 to 0.002 N), delivered from a volumetric burette.

We are greatly indebted to Dr. D. A. MacInnes for the design of the differential titration apparatus which made the attainment of the required accuracy for this work possible. The apparatus is shown, diagrammatically, in Fig. 1. Two spiral electrodes (*A* and *B*) of the type described by MacInnes and Belcher[7] were used. The electrode (*A*) forms part of a gas lift pump for circulating the titrated solution. A jacket of Jena glass, sealed around this electrode, is filled with 0.1 N hydrochloric acid solution from which a silver-silver chloride electrode (*E*) makes contact to one terminal of the potentiometer. Another, larger, spiral electrode (*B*) entirely encircles the smaller "pump" electrode (*A*). It is filled with 0.1 N hydrochloric acid from which another silver-silver chloride electrode (*F*) completes the circuit to the potentiometer. The outer electrode (*B*) is rigid enough to be used as a hand-operated stirrer. Commercial nitrogen, after passing over soda-lime and through a cotton filter, enters through the stop-cock (*S*), operates the gas lift pump, and escapes at (*O*). Thus, solution is drawn up at (*M*) and is passed through the inner part of the spiral (*A*), returning to (*N*). The differential titrations were carried out with a student type Leeds and Northrup potentiometer and a vacuum tube (pliotron) electrometer.[8]

The potential differences corresponding to values near the end-point for a 2-drop addition of 0.001 N HCl amounted, in the case of the dilute guaiacol solutions, to

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[5] The preparation of the salts and the purification of guaiacol have been described in the previous paper. Conductivity water was used for the aqueous phase.

[6] MacInnes, D. A., and Dole, M., *J. Am. Chem. Soc.*, 1929, **51**, 1119.

[7] MacInnes, D. A., and Belcher, D., *Ind. and Eng. Chem.*, *Analytical Edition*, 1933, **5**, 199.

[8] Hill, S. E., *Science*, 1931, **73**, 529. We are indebted to Dr. S. E. Hill for putting this instrument at our disposal.

11–12 mv., and to 3–5 mv. in the case of the more concentrated solutions. It was thus always possible to estimate the end-point within 2 drops of 0.001 N acid.

The results of the partition experiments for potassium guaiacolate are given in Table I and those for sodium guaiacolate in Table II. No corrections were made for hydrolysis in the aqueous phase, since it was shown previously that the errors arising from this source were less than the probable experimental errors.

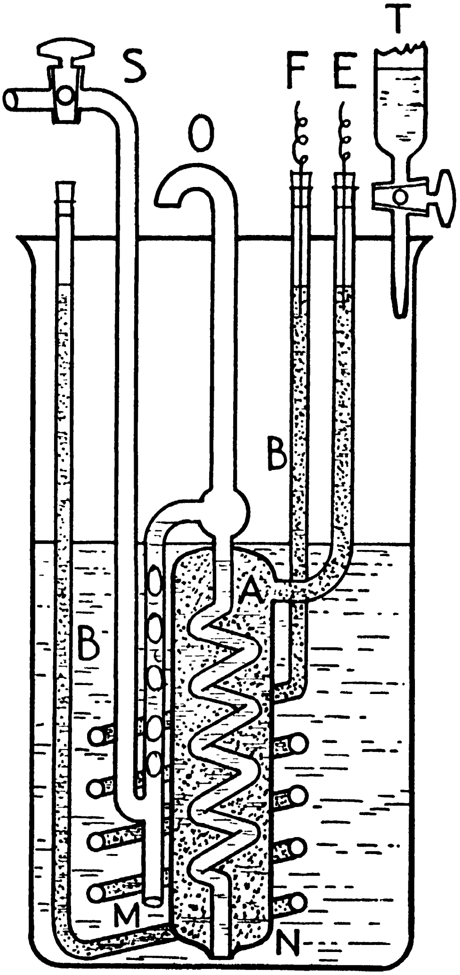

FIG. 1. Glass electrode differential titration apparatus

The method for calculating values of the dissociation constants of the alkali guaiacolates in water-saturated guaiacol has already been discussed in this paper.

TABLE I

The Distribution of Potassium Guaiacolate between Water and Guaiacol at 25°C.

Non-aqueous phase		Aqueous phase		Partition coefficient
C (moles/liter)				
Observed	Calculated*	C_1(moles/liter)	$C_1(\gamma_1)^2$	$S = \frac{C}{C_1}$
0.0613	0.0594	0.1397	0.0860	0.439
0.03247	0.0322	0.1001	0.0643	0.324
0.02037	0.02037†	0.0780	0.0517	0.261
0.01073	0.01081	0.0550	0.0381	0.195
0.00924	0.00925	0.0505	0.0353	0.183
0.00283	0.00290	0.02652	0.0199	0.107
0.00093	0.00090	0.01347	0.0108	0.0690
0.00055	0.00052	0.00974	0.00802	0.0566
0.00034	0.00029	0.00683	0.00577	0.0490

* $K = 5.1 \times 10^{-5}$.

† Reference point, $\beta = 4.61$; $S_0 = 0.0153$.

TABLE II

The Distribution of Sodium Guaiacolate between Water and Guaiacol at 25°C.

Non-aqueous phase		Aqueous phase		Partition coefficient
C (moles/liter)				
Observed	Calculated*	C_1(moles/liter)	$C_1(\gamma_1)^2$	$S = \frac{C}{C_1}$
0.005575	0.00559	0.06437	0.0437	0.0866
0.002780	0.00283	0.04396	0.0312	0.0632
0.002789	0.00282	0.04384	0.0312	0.0636
0.001702	0.001702†	0.03285	0.0241	0.0518
0.000998	0.00099	0.02408	0.0183	0.0415
0.000518	0.00052	0.01585	0.0125	0.0327
0.000344	0.00031	0.01189	0.00963	0.0290

* $K = 4.3 \times 10^{-5}$.

† Reference point, $\beta = 1.75$; $S_0 = 0.00855$.

In computing the activity coefficients for the ions, according to the Debye-Hückel theory (equations (3) and (3′)), a radius of 7 Å. has been assumed. This value was estimated with the aid of crystal structure data,[9] and is sufficiently accurate since it enters as a correction term in the equations.

For the aqueous phase, saturated with guaiacol, the dielectric constant is 76.1, and for the non-aqueous phase, saturated with water, it is 14.3.[10] Accordingly,

$$\log_{10} \gamma_1 = -\frac{0.526 \sqrt{C_1}}{1 + 2.32 \sqrt{C_1}}$$

and

$$\log_{10} \gamma = -\frac{6.52 \sqrt{C\theta}}{1 + 5.36 \sqrt{C\theta}},$$

that is, $a = 6.52$, $b = 5.36$, $a_1 = 0.526$, and $b_1 = 2.32$ in equations (3) and (3′).

In Fig. 2, for potassium guaiacolate and sodium guaiacolate respectively, the solid lines show plots of the partition coefficients S (ordinates) against $C_1\gamma_1^2$ (abscissae), C_1 referring to the salt concentration in the aqueous phase and γ_1 to the corresponding activity coefficients. The dotted lines in the figure show plots of $S(1 - \theta)$ against the same abscissae. It will be recalled that to compute values of θ, successive values for the limiting partition coefficient S_0 are assumed; and the correct value of S_0 is the one that sends the line, $S(1 - \theta)$ *vs.* $C_1\gamma_1^2$, through the origin. The slope of this line is $\frac{(S_0)^2}{K} = \beta$, from which K, the ionic dissociation constant, is computed.

The calculated results for potassium guaiacolate give values of $K = 5.5 \times 10^{-5}$ and $S_0 = 0.016$, and for sodium guaiacolate $K = 3.5 \times 10^{-5}$ and $S_0 = 0.007$. These values of K are in satisfactory agreement with those obtained from conductivity measurements, which were $K = 5.1 \times 10^{-5}$ for potassium guaiacolate, and $K = 4.3 \times 10^{-5}$ for sodium guaiacolate.

The agreement may, perhaps, be shown more clearly in another way. Equation (4) may be rewritten in the form

$$C = \beta C_1^2(\gamma_1)^2 + \theta C \tag{4′}$$

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[9] Pauling, L., *J. Am. Chem. Soc.*, 1927, **49**, 765.

[10] We are indebted to Dr. S. O. Morgan of the Bell Telephone Laboratories for these values.

We shall assume that K has the value determined from the conductivity measurements for each salt. Now, taking the measured values of C and C_1 for one partition experiment (a reference point), we calculate β in equation (4′), using equations (1) and (3). Taking this value of β, we then compute values of C corresponding to all the measured values of C_1. A comparison between the values of C (the alkali guaiacolate concentration in the non-aqueous phase) calculated in this manner and those obtained experimentally is shown in columns (2) and (1) respectively, of Tables I and II. The agreement is evidently quite satisfactory.

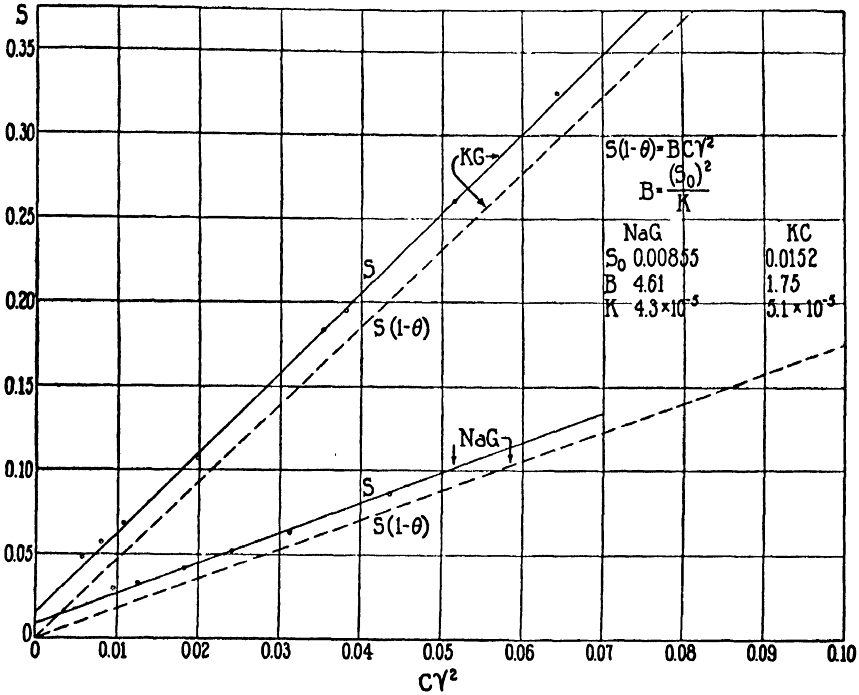

FIG. 2. Partition coefficients (S) of sodium and potassium guaiacolates between guaiacol and water at 25°C.

The Theoretical Prediction of Limiting Partition Coefficients

The relative concentrations of a solute species in two phases which are in equilibrium depend on the difference of the potential energy of the solute in the two phases. In this discussion, we shall confine ourselves to the simplest case (infinite dilution) for which the concentration ratio is the limiting partition coefficient, S_0.

It has been shown by Born,[11] from classical electrostatic theory, that the difference in potential energy resulting from the transfer of a spherical ion from one solvent to another is given by the equation

$$\Delta E = \frac{e^2}{2r}\left(\frac{1}{D} - \frac{1}{D_1}\right) \tag{8}$$

in which ΔE is the charge of potential energy, e is the electrostatic charge of the ion, r is the ionic radius, and D and D_1 are the dielectric constants of the two solvents. Furthermore, the Maxwell-Boltzmann distribution theorem gives us an expression for the concentration ratio:

$$\frac{C}{C_1} = \exp.^{-\frac{\Delta E}{kT}} \tag{9}$$

K is the Boltzmann constant, and T the absolute temperature.

Combining these two equations, we obtain

$$\log_e \frac{C}{C_1} = \frac{e^2}{2rkT}\left(\frac{1}{D} - \frac{1}{D_1}\right) \tag{10}$$

corresponding to the transfer of one kind of ion. However, since the electrical neutrality of the phases must be preserved, it is necessary to consider the simultaneous transfer of a negative and positive ion. Consequently, instead of equation (10) we have, for a simple uni-univalent electrolyte

$$\log_e \left(\frac{C}{C_1}\right)^2 = \log_e S_0^2 = \frac{e^2}{2kT}\left(\frac{1}{D} - \frac{1}{D_1}\right)\left(\frac{1}{r^+} + \frac{1}{r^-}\right)$$

in which r^+ and r^- are the respective radii of the positive and negative ions. This equation reduces to

$$\log_e S_0 = \frac{e^2}{4kT}\left(\frac{1}{D} - \frac{1}{D_1}\right)\left(\frac{1}{r^+} + \frac{1}{r^-}\right) \tag{11}$$

In the derivation of equation (11) it has been assumed that the properties of the solute depend only on its electrostatic charge and radius, and that those of the solvents depend on their dielectric constants alone. This means that all energy changes due to electrostriction, electrical saturation of solvent about the ions, ionic field asymmetries, electronic displacements, etc., have been neglected.

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[11] Born, M., *Z. Phys.*, 1920, **1**, 45.

Since most of the factors neglected in this simple treatment are concerned with forces operating close to the ions, we shall make the following additional assumption. Let us assume that most of the "close range" forces are confined to a region corresponding to a single layer of solvent molecules polarized around the ions. It will be recalled that the concentration of water in the guaiacol phase, saturated with water, corresponds to a mole fraction of about 25 per cent. Water molecules, although smaller in size, have larger electrical dipole moments than guaiacol molecules. It is therefore reasonable to suppose that even in the guaiacol phase the ions will be preferentially "solvated" with water.

We shall assume, then, that for the case of salts distributed between guaiacol and water, the ions may be regarded as similarly hydrated with a single sheath of water molecules in both phases.

Returning to equation (11), and substituting the proper numerical values ($k = 1.37 \times 10^{-16}$, $e = 4.77 \times 10^{-10}$, $T = 298°$ (for 25°C.), $D = 14.3$, $D_1 = 76.1$) we obtain

$$\log_{10} S_0 = -3.44\left[\frac{1}{r^+} + \frac{1}{r^-}\right] \tag{12}$$

in which, now, r^+ and r^- are the radii, in Ångstrom units, of the *solvated* positive and negative ions. These values we shall obtain by adding 1 Å. (for the water sheath) to the crystallographic ionic radii.[9] Thus $r_{\mathrm{Na}^+} = 1.95$ Å., $r_{\mathrm{K}^+} = 2.33$ Å., $r_{G^-} = 7.0$ Å. Substituting these values in equation (12) for sodium guaiacolate (Na*G*) and potassium guaiacolate (K*G*) we obtain:

$$S_0\,(\mathrm{Na}G) = 0.0055;\ S_0\,(\mathrm{K}G) = 0.011$$

which are in as good agreement as can be expected with the experimentally determined values:[12]

$$S_0\,(\mathrm{Na}G) = 0.007;\ S_0\,(\mathrm{K}G) = 0.016$$

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[12] Instead of equation (11) N. Bjerrum (*Tr. Faraday Soc.*, 1927, **23**, 447, 449) (see also *Z. phys. Chem.*, 1927, **127**, 358; 1932, **159**, 194) has used equation (10), with a value of r equal to the mean of the positive and negative ionic radii, and has shown that it fails to predict actual partition coefficients.

It is thus possible to predict from equation (12) that the smaller the ion sizes the smaller will be the limiting partition coefficients (S_0). The stoichiometric partition coefficient (S) (see equation (4)) is directly proportional to the square of (S_0), and indirectly proportional to the dissociation constant (K).

It can be shown[13] that the same factors which increase (S_0) also increase (K). However, since S is proportional to the first power of $\left(\frac{1}{K}\right)$ and to the square of (S_0), the latter primarily determines the change of (S) with the concentration. Thus we may conclude that the slope $\beta = \frac{(S_0)^2}{K}$ of equation (4) will be larger for salts having larger ions. This generalization is confirmed in the recent partition experiments of Osterhout, Kamerling, and Stanley,[14] who found that for the alkali guaiacolates distributed between water and guaiacol-*p*-cresol mixture, S increases in the order Cs > Rb > K > Na > Li, which is precisely what we should expect from the corresponding ion radii. The small partition coefficient for potassium chloride, measured by the same investigators can similarly be predicted from equation (12), since the "hydrated" chloride ion ($r_{Cl^-} = 2.87$ Å.) is considerably smaller than the corresponding guaiacolate ion ($r_{G^-} = 7.0$ Å.).

The effect of temperature on partition coefficients can be determined from equation (11). Since the logarithm in this equation is numerically negative, we can predict a greater partition coefficient at a higher temperature.

SUMMARY

1. Measurements are reported on the distribution of sodium and potassium guaiacolates between guaiacol and water at 25°C.

2. The variation of the partition coefficients with the concentration is explained with the aid of the Debye-Hückel interionic attraction theory and the assumption that the salts are strong electrolytes in water and weak electrolytes in guaiacol.

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[13] Bjerrum, N., *K. Danske Vidensk. Selsk.*, 1926, **7**, No. 9. Fuoss, R. M., and Kraus, C. A., *J. Am. Chem. Soc.*, 1933, **55**, 1019.

[14] Osterhout, W. J. V., Kamerling, S. E., and Stanley, W. M., *J. Gen. Physiol.*, 1933–34, **17**, 469.

3. The dissociation constants of sodium and potassium guaiacolates in guaiacol previously computed from electrical conductivity determinations are shown to be in agreement with the corresponding values obtained from the distribution measurements.

4. From theoretical considerations an equation is derived with which it is possible to predict the magnitude of the limiting partition coefficients from the dielectric constants of the solvents, the size of the solute ions, and the temperature.

INFLUENCE OF DEATH CRITERIA ON THE X-RAY SURVIVAL CURVES OF THE FUNGUS, NEUROSPORA*

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Most recent papers on the effects of X-rays on microorganisms have been based on quantitative investigations, and the results usually have been expressed in the form of survival curves where the survival ratios are plotted as a function of the X-ray dosage. In general, attempts have also been made to correlate the findings with the predictions of the quantum hit theory. Originally derived in an elementary form by Crowther (1926) and Condon and Terrill (1927) from statistical considerations of the quantum nature of radiation, this theory has recently been elaborated by Glocker (1932) so as to be applicable to more complex biological systems.

The quantum hit theory has tacitly assumed that the concept of death is absolute and precise—that a distinction between the condition of life and death in a given organism can always be made. In determining survival ratios, one must first select some reliable criterion as to what constitutes survival. No theoretical significance can be attached to survival curves obtained in the absence of an accurate standard. For example, the traditional index of death adopted by bacteriologists is the failure of a cell to produce a macroscopic colony, or, in other words, the loss of the reproductive function.

The shape and meaning of bacterial killing curves have been discussed by Rahn (1932). It has been customary to draw such graphs on semilogarithmic paper.

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* The authors take pleasure in expressing their gratitude to Dr. Lee Bonar for the privilege of frequent consultation and advice, and to Dr. T. H. Goodspeed for his enthusiasm and generosity in placing at our disposal his facilities for research with X-radiation.

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When data on the bactericidal effects of X-rays were graphed in this orthodox manner by Holweck and Lacassagne (1929, 1930*a*) and Wyckoff (1930), straight lines were obtained. These were immediately interpretable in terms of the quantum hit theory with which they were in complete agreement, and meant that a bacterium was killed by the absorption of a single quantum. Since it was known from energy considerations that each bacterium had actually absorbed many quanta, the particular one effective in causing death was considered to have been absorbed in a so called sensitive volume. Such sensitive regions have been assigned values ranging from cell nuclear to molecular dimensions, depending on the experiment. Although no effects have been ascribed to the numerous quanta absorbed outside the sensitive volume, Wyckoff (1932) has recently had some misgivings about them.

Additional experiments to prove the correctness of the quantum hit theory were soon under way with yeasts as the test organisms. Life was found to continue even though the budding process was inhibited; furthermore, many cells were found budding one or more times before succumbing to apparent death. Although reproducible survival curves were obtained by Holweck and Lacassagne (1930*b*), Wyckoff and Luyet (1931), and Glocker, Langendorff, and Reuss (1933), the adoption of arbitrary death criteria permitted no unique interpretations in terms of the theory. Rahn and Barnes (1933) in an experimental study of death criteria in yeast found their curves radically affected by the following selection of standards: reproduction, staining, rate of CO_2 production, and protoplasmic coagulation.

Very interesting in this connection are the results obtained by Zirkle (1932) on fern spores irradiated with alpha particles. Each of the three death criteria he used yielded a different value for the number of hits required to kill.

Of the several references known to the authors on the effects of X-radiation on fungi, only two contain quantitative data. Attempts by Leonian (1929) and Heldmaier (1929) were entirely negative; Holweck and Lacassagne (1930*b*) found their material unsuitable for quantitative study; Nadson and Philippov (1925) report qualitative observations only. Studies on the control of agriculturally important fungi with X-radiation have been reported by Tascher (1933). Although most of his paper is devoted to a study of induced sector mutations, Dickson (1932) has published a killing curve for *Chaetomium cochliodes*, plotting number of injured spores against the dosage. The difficulties encountered by Luyet (1932) in his studies with the fungus, *Rhizopus nigricans*, are partially revealed in the following quotation: "Besides the giant spores without mycelia, others are found which, growing short mycelia before their development ceases, represent all transition stages toward normal cells. . . . . The presence of swollen spores obviously makes it difficult to set up the accurate criterion of death necessary for obtaining significant survival ratios."

In inaugurating the researches reported in this paper, the authors were desirous of finding a fungus suitable for a quantitative study of certain biological effects of X-rays. Two species of the ascomycetous fungus *Neurospora*, *N. sitophila* and *N. tetrasperma*, were selected. These are closely related species which do not hybridize freely, but as the former has uninucleate ascospores and the latter binucleate, some variation in the shape of their survival curves or in the rate of induced mutations was considered possible.

The genus *Neurospora* is well known from the papers of Shear and Dodge (1927), Dodge (1927, 1928, 1931), and Lindegren (1932, 1933). *N. sitophila* produces 8 unisexual, uninucleate, haploid ascospores in each ascus. A single spore culture gives a vegetative colony which is genetically homogeneous, for all the cells have been obtained from a haploid cell. Since these single spores are easy to isolate, this is a particularly favorable organism in which to study mutation rates in either control or irradiated cultures. Since the vegetative cultures are haploid, there is no problem of dominance for the vegetative characters; therefore, backcrossing is unnecessary. This may not be true in a study of reproductive characters. Sexual cultures of *N. sitophila* may be obtained readily by breeding the two sexually allelomorphic strains. *N. tetrasperma* produces 4 binucleate, bisexual, diploid spores in each ascus. If there is any dominance, the frequency of apparent variation should be much less. This organism is probably not so suitable for a study on the rate of mutation; however, it is more convenient for determining the effects of X-rays on sexual reproduction.

X-Radiation

As a source of low-voltage X-radiation, a water-cooled Mueller tube (the so called Grenz ray tube) was employed. In this particular form of tube, the filament leads extend through a hole in the center of the anticathode so that the focal source is annular in shape and inclined at an angle of 45° with respect to the utilizable X-ray beam. The 60 cycle alternating potential applied to both tube and filament was supplied by a manually controlled Wappler installation. Throughout the course of the experiments, the calibrated voltmeter on the primary of the high voltage transformer read 11 kv. (root mean square). The tube current was held constant at 8 milliamperes. Although no spectral analysis has been made of the quality of the radiation, it seems clear from the data of Glassner (1932) and Exner (1932) that over 95 per cent of the measured intensity was included in the wave length range from 1 to 2.5 Ångström units. The Lindemann glass window in the tube served as the only filter.

Dosage measurements in terms of the international roentgen were carried out with an open air ionization chamber and accessory apparatus previously constructed by one of the authors. Though similar in design to the instrument of Taylor and Stoneburner (1932), it differs in a number of structural features which have been described elsewhere by Uber (1933). Except in the germination experiments, the intensity used throughout was such as to give a measured dosage of 210 roentgens per minute. This value would be increased somewhat by scattered radiation.

Uniformity of X-ray intensity in a radial direction for the effective beam was found by ionization measurements to lie within the limits of the experimental error. Angular uniformity was secured by rotating the irradiated material continuously on a clinostat, the period of rotation being small compared to the exposure times. Since it was impossible to eliminate scattered radiation, an attempt was made to have its contribution to the total intensity a constant one over the area used. To accomplish this, the material was placed on a smooth agar surface which in turn was laid on a plane sheet of lead resting on the clinostat.

Material and Methods

Cultures of *Neurospora sitophila* and *N. tetrasperma* were kindly furnished us by Dr. B. O. Dodge of the New York Botanical Garden. Strains of the former bore the markings 56:8 A and 56:3 B, the latter 19.3 e. Both species produce an abundance of black ascospores which are discharged from their perithecia when mature. These spores are approximately 13–15μ in diameter and 23–31μ in length, the upper limits in each case being characteristic of *N. tetrasperma.* Conidia and microconidia are also produced. The prolonged heat treatment, to which the ascospores must be subjected in order to initiate growth processes, insures purity of the cultures by killing vegetative cells or contaminating organisms. This obviation of sterility precautions makes these ascospores very desirable test objects from the standpoint of experimental technic.

A suspension of ascospores, which had been discharged from mature perithecia, was made in distilled water. The dilution of the suspension was adjusted by trial so that when the surface of a circular agar disc was flooded the spores were well separated. The plain 3 per cent agar was 15 cm. in diameter and 3 mm. thick. After the surplus water had evaporated from the surface, this sheet of agar and its lead support were centered on the clinostat. The irradiation was carried out with the spores 21 cm. from the effective focal plane of the anticathode. Angular sectors of the agar disc were removed at the end of the several exposure intervals. When all of the exposures had been made the irradiated sectors and the controls were subcultured.

Single spore inoculations were made from the various sectors and controls with the aid of dissecting microscopes and small spatulas. From 100 to 150 single spore isolations were made for each interval, and each spore was inoculated into a 10 cm. test-tube. The nutrient medium was 0.5 per cent malt extract and 0.5 per cent glucose in 2 per cent agar.

The heat treatment administered to these agar slant cultures was carried out in a thermostat oven at 60°C. After exposure periods ranging from 1½ to 2½ hours, depending on the particular experiment, the cultures were transferred to the 25°C. incubation room.

Qualitative Results

Early experiments with *N. sitophila* were largely of an exploratory nature. In group germination studies of the irradiated ascospores, a marked delay in growth ranging from several hours to several days was at once apparent. Development of the spores in the higher dosage groups soon ceased, only short germ tubes having been produced. With successively shorter exposures, there was a progressive increase in the mycelial growth which finally obscured all observations. That the really interesting phenomena were being masked and that it would be necessary to adopt some such method as the one outlined in the preceding section were immediately evident.

Since each spore gave rise to an individual test-tube culture, the fate of each one could be easily followed. Most striking was the large percentage of abnormal cultures which developed from the irradiated spores while the controls showed almost complete uniformity; however, the abnormalities were practically continuous in gradation. Among the many mutant characteristics, the following may be mentioned: color variations in conidial masses, discoloration of agar substrate, abortive perithecia, non-conidial albino strains, unusual types of aerial mycelium, and the so called "wet" cultures. These qualitative observations parallel those of the earlier investigators on fungi.

Survival curves for *N. sitophila* were obtained too early in the evolution of our technic to be of quantitative value. In cases where the data are comparable, they corroborate the results obtained with *N. tetrasperma.*

Quantitative Results

Group Cultures.—The methods used in obtaining quantitative data on ascospore germination differ in several particulars from those outlined on previous pages. Higher dosage requirements necessitated shortening the distance from the anticathode to the irradiated material from 21 cm. to 9 cm. The approximate dosages for each of the exposure periods, as given in Table I, are to be considered as minimal values. Upon removal from the X-ray beam, the original sectors of the agar disc holding the spores were placed in Petri dishes; at the conclusion of the series of exposures, these were heated for 2 hours in a thermostat oven at 62°C. The germination counts were made 12 hours later, the protrusion of as much as a mycelial beak being considered as an index of germination.

It will be noted from Table I, and from Table II as well, that control germinations under the conditions of the experiment were unusually uniform—ranging from 92 to 95 per cent. Series H and K differ in that the spores in Series K were a month older; the latter were obtained from a culture aged 3 months. When presented in graphical form as Fig. 1, these results exhibit several striking features. Outstanding is the existence of a low minimum in the survival curve followed by a high secondary maximum. As shown by the composite growth curve which has been inserted for comparison, the normal growth process is inhibited long before the mechanism responsible for germination is destroyed. No relationship whatsoever seems to exist between germination and growth, other than that the former is necessarily a prerequisite of the latter.

TABLE I

Germination Data for X-Irradiated Ascospores of N. tetrasperma

X-ray exposure	Approximate dosage	Series H		Series K	
		No counted	Germinated	No counted	Germinated
hrs	*roentgens*		*per cent*		*per cent*
0	0	244	93 5	804	95
1	70,000	228	91 7	545	92 4
2	140,000	217	76	347	85
2½	175,000			368	83
3	210,000	600	00	510	51
3½	245,500			588	34 5
4	280,000	168	46	653	82
5	350,000	373	93	446	26
6	420,000	203	94	788	2 5
7	490,000	174	79		
8	560,000	162	59		

With increasing X-ray dosage up to 100,000 r., there is a progressive decrease in the length of the germ tube. This is in agreement with the observations of Dickson (1932) on *Chaetomium cochliodes* and Luyet (1932) on *Rhizopus nigricans*. It is difficult to determine just what constitutes germination at the higher doses. Spores receiving dosages around 150,000 r. produced short, swollen germ tubes. Upon closer examination these were found to contain coagulated protoplasm and were obviously dead. For dosages around 300,000 r. or more, the germ tubes were quite transparent as if filled with an aqueous solution. This is the region of the second maximum in the germination curve.

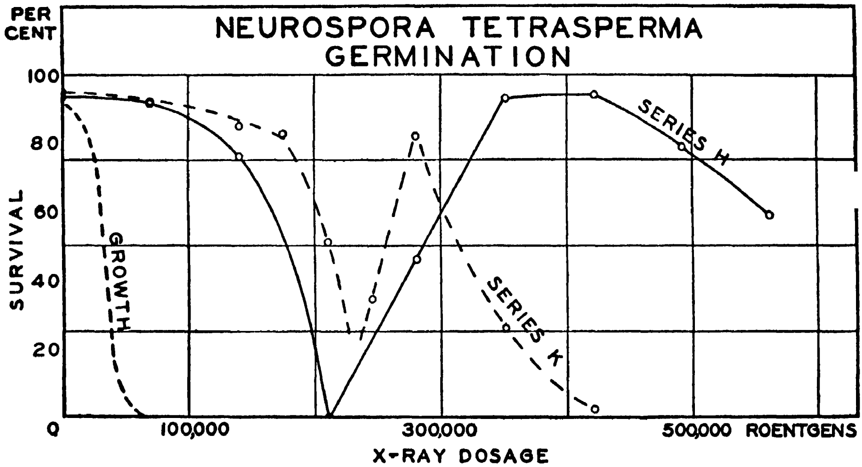

FIG. 1. Survival of irradiated ascospores using germination as a criterion of death. Note that growth ceases at a comparatively low dosage.

None of these germ tubes grew appreciably in length, and it seems probable that all these spores, even though producing germ tubes, were dead. It appears likely that the germination curve is dependent upon certain physical rather than physiological effects of the X-rays. The germination curve, then, probably represents the course of certain physical changes in a non-living system. Spore germination is not a criterion of the life of the cell; therefore, it cannot be used for obtaining data with which to test the quantum hit theory of lethal action. Luyet (1932) has used spore germination for such a criterion; from the above discussion this seems not to be justified.

Single Spore Cultures.—The data on single spore cultures of irradiated *N. tetrasperma* were obtained according to the methods already described in a previous section. The results are tabulated in Table II. Series E and G differ in regard to their heat treatment, the former being heated 1½ hours and the latter 2½ hours at 60°C. It was considered highly desirable to avoid all artificial distinctions in ascertaining the lethal effects produced. Consequently, only those criteria of death which appeared to have a real significance in the life cycle of the organism were employed. These were germination, positive growth, mature ascospores, and the production of normal perithecia. Perithecia have been considered normal if they discharged their spores within 3 weeks after irradiation. In determining the cultures with mature ascospores, the non-discharging perithecia were crushed on cover-glasses and examined under the microscope. By "positive growth" is meant cultures whose mycelia covered the

TABLE II

Single Spore Culture Data for X-Irradiated N. tetrasperma
(Series E Above; Series G Below)

Exposure time	Total No. of cultures	No. of cultures with					Cultures with	
		Normal perithecia	Mature ascospores	Perithecia absent or sterile	Positive growth	Growth absent	Positive growth	Mature ascospores
hrs.							*per cent*	*per cent*
0.0	100	87	88	4	92	8	92	88
0.5	100	84	87	1	88	12	88	87
1.0	60	38	49	2	51	9	85	82
1.5	149	31	73	35	108	41	72	49
2.0	150	7	30	80	110	40	73	20
3.0	149	0	2	20	22	127	15	1.3
4.0	100	0	0	1	1	99	1	0
5.0	97	0	0	0	0	97	0	0
0.0	100	93	93	1	94	6	94	93
0.5	100	79	79	2	81	19	81	79
1.0	99	77	88	3	91	8	92	89
1.33	145	63	98	20	118	27	81	68
1.66	150	33	64	74	138	12	92	43
2.0	149	3	19	105	124	25	83	13
2.5	100	1	4	28	32	68	32	4

surface of the agar substrate and/or which were capable of being subcultured. The selected groupings were found to possess well defined natural boundaries. Within each classification, however, continuous gradations existed. For example, the class "mature ascospores" includes those cultures which discharged spontaneously, those which contained a full complement of spores but did not discharge, and finally perithecia which contained relatively few mature spores. Similarly, under "perithecia absent or sterile" are grouped conidial and non-conidial cultures along with those having scleroid bodies, abortive perithecia, etc. The appearance of 4 non-perithecial cultures in the Series E controls is probably due to the unintentional culturing of small uninucleate spores. Since it was known that a small percentage of such spores appears in *N. tetrasperma*, an effort was made to select only the uniform binucleate variety. Even the binucleate spores may be unisexual in rare cases (see Lindegren, 1932). The final counts were made 30 days after the date of irradiation. That equally significant data might have been secured at a much earlier date was indicated by superficial counts made in the interim.

A graphical presentation of much of the data in Table II is furnished in Figs. 2 and 3, though in a slightly altered form. The survival ratios have been adjusted for the controls by setting the value of the latter at 100 per cent. In order that all the curves might be readily comparable with each other, the X-ray dosage in roentgens has been plotted as the abscissa in each case. In Figs. 2 and 3, each abscissa division corresponds to a time period of 1 hour or a dosage of 12,500 r. The germination curve, which for such low dosages is practically a straight line, has been sketched for comparison. No graphs have been drawn to represent the survival of normal perithecia since the values to be plotted for such curves are almost identical with the ascospore ratios for the initial periods of exposure; however, at the higher dosages the development of normal perithecia was curtailed very markedly. Thus while both nuclei of a spore might survive the irradiation process, injury sufficient to interfere with the automatic dispersal of mature ascospores from the perithecia was frequently sustained.

The dosages corresponding to 50 per cent survival for ascospore production and growth are approximately 20,000 and 30,000 roentgens,

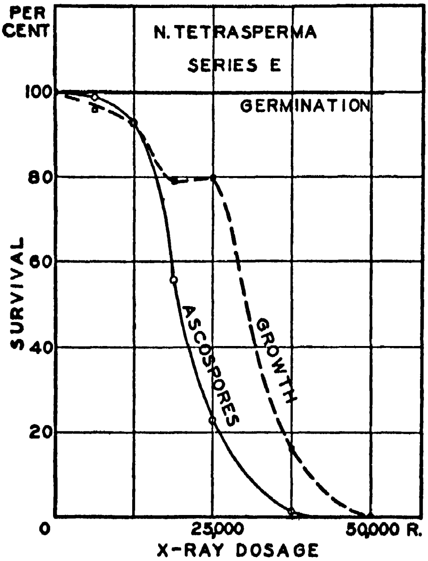

FIG. 2. Survival curves for irradiated ascospores, showing the dependence of shape on the choice of death criteria.

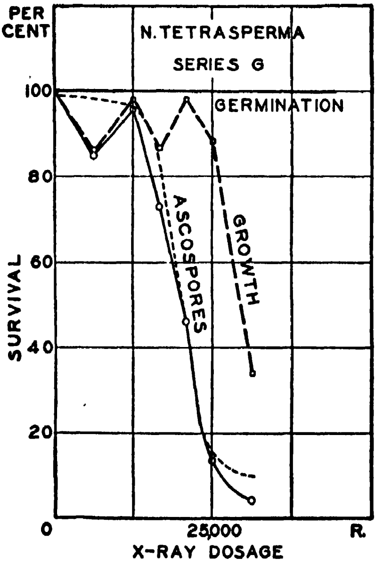

FIG. 3. Survival curves for irradiated ascospores. The broken line represents the ascospore production expressed as a percentage of the number of growing cultures.

respectively. Since two nuclei must be operative in sexual reproduction while only one may suffice for growth, one would expect from probability considerations some such relationship between these half-survival dosages. For example, if the probability of one nucleus in a spore being mortally injured by a given dosage is 0.5, the chance of both suffering the same fate is the product of the separate probabilities, or 0.25. This assumes that both nuclei are equally sensitive and that the theory of probability is valid when applied to the phenomenon of death induced by X-radiation. Though the above value fits the conditions in Fig. 2 rather well, the agreement with additional calculations made in the same manner is far from satisfactory in general.

The zigzag nature of the growth curve in Fig. 3 may be spurious, such as would result from sampling or other error. It is certainly brought about by a cause other than that immediately responsible for the processes of sexual reproduction. Support for such a view is seen in the shape which the ascospore curve for Series G assumes when the survival ratios are based on the number of positive growth cultures instead of on the total number of tubes inoculated. This transformation gives the broken curve in Fig. 3, a curve which is regular in shape and approximately congruent with the corresponding one in Fig. 2. Most of the data graphed in Figs. 2 and 3 are unsuitable for an analysis on the basis of the quantum hit theory due to irregularities in the shape of the curves. If this anomalous behavior is real, and due, perhaps, to the interaction of several destructive phenomena, the theory is not strictly applicable unless the gross survival curves can be resolved into their component parts. If one plots the standard theoretical survival curves corresponding to the effective number of hits required to kill (see Condon and Terrill, 1927), and compares them with the ascospore curve in Fig. 2, one obtains the value 9 for the best fit. From energy considerations it is now possible to calculate the so called sensitive volume of the spores. Assuming the effective wave length to be 1.5 Ångström units, the specific gravity 1.1, the ionizing energy in volts per ion pair 35, and making other customary assumptions as to absorption coefficients, etc. (see Wyckoff, 1930), one finds for the supposed sensitive volume a diameter of 0.5μ. Recalling that the ellipsoidal spores have the minor and major axes 15μ and

31μ respectively, one has a reference basis for comparison. In the absence of precise data on nuclear dimensions, it seems reasonable to assume that the sensitive region in volume corresponds to the chromatin or perhaps to a single chromosome. Since the X-rays were neither monochromatic nor filtered, any attempt to arrive at a more accurate picture of the lethal process with the data at hand would be manifestly unjustifiable.

DISCUSSION

Just as the life of a cell demands the presence and functioning of several distinct structures, so death processes may be induced by the destruction or injury of any one or all of these necessary cell entities. Hence one would not anticipate *a priori* a simple killing curve. That many survival curves do possess an elementary shape is probably attributable to the fact that some cell structures are much more sensitive than others to lethal agents. In his studies with fern spores, Zirkle (1932) has shown that the nuclear elements are much more susceptible to alpha radiation injuries than the extranuclear ones, but that sufficient interference with the functions of the latter will also destroy life. In fact, Zirkle found that the shape of his survival curves could be completely altered by changing the orientation of the spores with respect to the source of radiation—thus rendering the nuclei more or less accessible to the alpha particles. While such radical effects are not to be expected under the conditions of the present experiment, it is well to bear the possibility in mind when employing low voltage X-rays.

The fact that germination—defined as the protrusion of microscopically detectable material from the spore—can withstand extremely high dosages compared to the mechanism involved in growth, indicates that the two processes are quite dissimilar or at least proceed at very different rates. It is known that the continued growth of an organism requires a nucleus, although cytoplasm may live for some time without the benefit of such a structure. Increasing degrees of cytolysis under prolonged X-radiation might therefore account for decreasing mycelial development with exposure time. It is very probable, too, that germination phenomena are intimately associated with structural changes in the spore membrane. The question of permeability assumes importance in connection with osmotic phenomena. That X-radiation renders cell membranes more permeable to many solutions has been established by several investigators (see Kovacs, 1928). Diffusion coefficients likewise increase.

Along with these considerations, it is interesting to read the recent papers of Nakashima (1926) and Rajewsky (1930) on the effects of ultraviolet light and X-rays in coagulating pure proteins *in vitro.* They obtained a rhythmic curve for the number of protein particles observable in the ultramicroscope when plotted as a function of dosage. Just how far such a picture may be applicable, if at all, to the data on *Neurospora* germination cannot be foretold.

Recent studies by Stubbe (1933) on the variation of mutation rates in *Antirrhinum majus* with X-ray dosage may possibly have a bearing on this problem. Over a wide range of X-ray wave lengths, Stubbe found with increasing dosage that the mutation rate increased to a maximum, then decreased to a minimum, and finally increased again.

SUMMARY

1. When ascospores of *Neurospora tetrasperma* were irradiated with 11 kv. X-rays, the single spore cultures obtained displayed a wide variety of mutated forms.

2. Control germinations of ascospores showed uniform behavior, ranging from 92–95 per cent germination.

3. The shape of the survival curves was found to be a function of the criterion of death. The following criteria were used: germination, growth, production of mature ascospores, and the production of normal perithecia.

4. The germination survival curve exhibited a rhythmic variation with dosage. Germination is not a significant criterion of death.

5. Half-survival dosages for growth and ascospore production were approximately 30,000 and 20,000 roentgens, respectively.

6. Multiple hit-to-kill relations were found on the basis of the quantum hit theory; no accurate analysis was possible.

7. The studies indicate that ascospore death does not result from a single well defined reaction, but rather from the integrated effects of several deleterious processes initiated by the radiation.

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INACTIVATION OF CRYSTALLINE TRYPSIN

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Trypsin, like other enzymes, becomes inactive when in solution, and the rate at which this inactivation occurs depends upon the temperature, the pH, the concentration and the purity of the solution. Crude trypsin preparations have a maximum stability at about pH 5.0 and the solutions become more stable as the concentration is increased (1). They are completely and permanently inactivated if heated above 70°C. Solutions of purified crystalline trypsin behave quite differently; they may be heated to boiling (2) (in acid solution) for a short time without permanent loss in activity; in slightly alkaline solution they become more unstable as the concentration of the enzyme increases.

Since the crystalline preparation is apparently a pure protein it is possible to follow changes in the composition of the solution as well as changes in activity. A detailed study has been made of the inactivation of solutions of crystalline trypsin at various pH ranges and at various temperatures below 37°C. in order to determine the nature of the reactions and to see whether the loss in activity under all conditions is proportional to the decrease in trypsin protein.

Reversible Inactivation

The inactivation of trypsin solutions may be either reversible or irreversible. Reversible inactivation is caused by raising the temperature or by making the solution strongly alkaline. This reaction is practically instantaneous. The loss in activity is accompanied by the appearance of reversibly denatured protein which is insoluble in 0.5 M salt solutions at pH 2.0 and which is inactive. The native active protein is soluble even in molar salt solutions at pH 2.0. This reversibly denatured protein is in equilibrium (3) with the active native protein and reverts to the active native protein if the solution is allowed to stand at pH 2.0 and at about 20°C. At 0°C. the reversibly denatured protein becomes demonstrable in the solutions at about pH 8.0. As the alkalinity is increased from pH 8.0 to pH 12.0 the percentage of the trypsin protein present in this reversibly denatured form increases rapidly and at pH 13.0 practically all of the enzyme is in the denatured form. This decrease in the active form of the enzyme present from pH 8.0 to 13.0 agrees quite closely with the decrease in the rate of digestion of proteins by trypsin in this range of pH. The formation of this denatured form of the enzyme accounts for the effect of the pH on the digestion of proteins with trypsin on the alkaline side of the optimum and offers experimental evidence to show that changes in the nature of the enzyme protein result in corresponding changes in activity.

Irreversible Inactivation

The rapid loss in activity at higher temperatures or in alkaline solutions, just described, is completely reversible for a short time only. If the solutions are allowed to stand the loss in activity becomes gradually irreversible. This irreversible inactivation is accompanied by the appearance of various reaction products the nature of which depends upon the temperature and pH of the solution. The loss in activity at various pH is shown in Fig. 1. On the acid side of pH 2.0 the trypsin protein is changed to an inactive protein which is irreversibly denatured by heat. The course of the inactivation in this range of pH is monomolecular. The rate of inactivation decreases as the acidity becomes less and is very slow at pH 2.0.

From pH 2.0 to about pH 9.0 the trypsin protein is slowly hydrolyzed and decomposition products which are not precipitated by trichloracetic acid (non-protein nitrogen) appear in the solution. The amino nitrogen content increases but no ammonia is liberated. The kinetics of the irreversible inactivation in this range of pH agree with the assumption that the active native protein hydrolyzes the denatured form with which it is in equilibrium. The irreversible inactivation is, therefore, bimolecular in this range of pH and the rate of inactivation increases from pH 2.0 to pH 10.0 and then decreases. Since the inactivation due to the formation of inactive protein increases with increasing acidity, while inactivation due to hydrolysis of the protein increases with increasing alkalinity, there is a pH at which the total inactivation is minimum. This point of minimum irreversible inactivation or point of maximum stability is at pH 2.3.

On the alkaline side of pH 13.0 the reaction is similar to that in strongly acid solution and consists in the formation of inactive protein. No non-protein products are formed during the inactivation and the course of the reaction is monomolecular. The velocity of the reaction increases with increasing alkalinity.

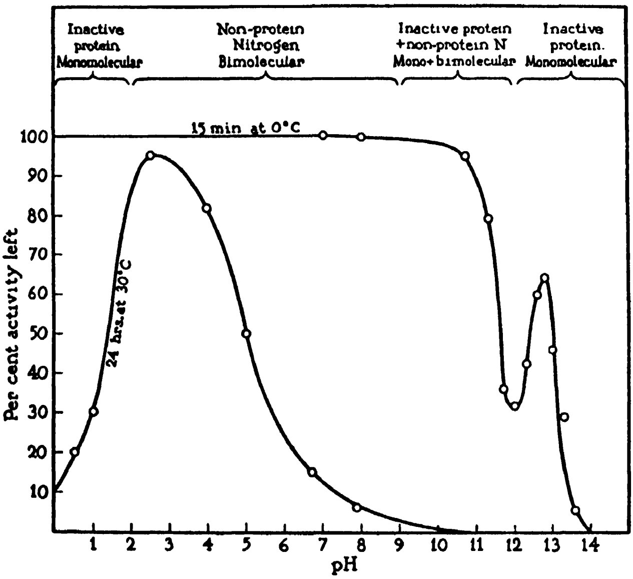

FIG. 1. Loss in activity of trypsin solutions at various pH

In the intermediate range of pH, from 9.0 to 12.0, the trypsin protein is partly hydrolyzed and partly changed to inactive protein so that the course of the reaction is represented by the sum of the bimolecular reaction, representing the hydrolysis, and the monomolecular, representing the transformation into inactive protein. As the pH is increased beyond 11.0 the percentage of active trypsin in solution decreases rapidly so that the rate of the reaction resulting in the hydrolysis of denatured trypsin becomes progressively slower. On the other hand, the reaction which results in the formation of inactive protein becomes progressively more rapid so that as a result of these two reactions there is a second point at about pH 13.0 at which the rate of irreversible inactivation is a minimum.

It was found in general that the decrease in activity under all the various conditions was proportional to the decrease in concentration of the trypsin protein although in strongly acid or alkaline solutions a small amount of inactive protein is formed at 30°C. which cannot be separated quantitatively from the trypsin protein. As a result the specific activity of the "active native protein" decreases during the experiment. In no case was the specific activity of the protein fraction higher than that of the original trypsin protein. There is, therefore, no indication that protein can be destroyed without a corresponding loss in activity. This result also shows that none of the split products of the trypsin protein has any appreciable activity. They are, therefore, similar to the results of the experiments on the inactivation of pepsin (4). Equations have been derived which agree quantitatively with the results of the various inactivation experiments.

EXPERIMENTAL RESULTS

It was found that inactivated trypsin solutions could be quantitatively analyzed for several definite fractions and these are referred to here as though they consisted of single compounds although they are probably mixtures of similar compounds. In order to describe the results in detail it is necessary to define these various fractions. The following terms have been selected for their brevity and are defined more or less arbitrarily in relation to the properties which distinguish the fractions during the analysis.

Total Protein.—Protein precipitable by 2.5 per cent trichloracetic acid at 75°C.

Total Native Protein.—Total protein soluble in M/1 sodium chloride or sodium sulfate at pH 2.0.

(*a*) "*Inactive Native Protein.*"—Native protein insoluble in cold salt solution after heating in salt-free acid. This fraction has no tryptic activity.

(*b*) "*Active Native Protein*" (*Trypsin Protein*).—Native protein soluble in cold salt solution after heating in acid. This fraction contains the tryptic activity.

Total Denatured Protein.—Protein insoluble in M/1 sodium chloride, pH 2.0.

(*a*) "*Irreversibly Denatured Protein.*"—Denatured protein which does not become soluble in molar salt solution after standing in dilute salt solution at pH 2.0.

(*b*) "*Reversibly Denatured Protein.*"—Denatured protein which becomes soluble in molar salt solution after standing in dilute salt solution at pH 2.0.

"*Potentially Active Protein.*"—Active native protein in the original solution plus reversibly denatured protein (*i.e.* protein which becomes active native protein in dilute salt-free acid during the analysis).

"*Inactive Protein.*"—Protein insoluble in cold M/1 sodium chloride, pH 2.0 after heating in salt-free acid solution; equals inactive native protein plus irreversibly denatured protein.

"*Activity.*"—The activity of the solution determined under conditions which prevent reactivation of any inactive enzyme (10 *a*). It is measured by the rate of hydrolysis of a standard hemoglobin solution and expressed in terms of trypsin units $[\text{T. U.}]^{\text{Hb}}$. One $[\text{T. U.}]^{\text{Hb}}$ produces in 1 minute at 35.5°C. in 6 ml. digestion mixture an amount of color-producing substance not precipitated by trichloracetic acid which gives the same color as 1 milliequivalent of tyrosine (10 *b*).

"*Potential Activity.*"—The activity of the solution after standing in dilute salt-free solution at pH 2.0. Under these conditions the reversibly denatured trypsin reverts to the active native form.

A. Reversible Inactivation of Trypsin in Alkaline Solution

1. Effect of pH.—If solutions of purified trypsin are heated the trypsin is denatured but returns to its native condition upon cooling. The effect of alkali is similar to that of heat. If a series of solutions of trypsin at 0°C. are titrated to increasingly alkaline reactions and samples taken immediately into cold salt solution at pH 2.3, the denatured trypsin protein precipitates. The per cent of the protein in this denatured form increases with the alkalinity until at pH 13.0 practically all of the protein is denatured and inactive. If samples are taken immediately into dilute salt-free acid so that the resulting solution is about pH 2.0 and these solutions are then allowed to stand for several hours, no precipitate appears upon the subsequent addition of salt and the activity of these samples is the same as the original total activity of the solution. The results of an experiment of this kind are shown in Fig. 2 in which the per cent denatured protein, as determined by analysis, or the per cent loss in activity, as determined by activity measurements, is plotted against the pH. The figure shows that the fraction of the protein present in the denatured form is equivalent to the fraction of the activity lost. It will be noted that the fraction of active native trypsin remaining in solution decreases rapidly on the alkaline side of pH 8.0 and follows quite closely the pH activity curve of trypsin-casein digestion. The decrease in the rate of digestion of proteins by trypsin as the alkalinity is increased beyond pH 8.0 is evidently due, therefore, to the formation of reversibly denatured trypsin. This result is direct experimental evidence confirming the hypothesis of Michaelis and Davidsohn (5) that the effect of pH on the activity of trypsin is due to an equilibrium between an active and an inactive form of the enzyme which is shifted toward the inactive side as the alkalinity is increased. It had previously been found that the increase in the rate of digestion of proteins by trypsin (6) as the alkalinity increases from pH 5.0 to 8.0 agrees with the assumption that the trypsin reacts with the negative protein ions. It is difficult on this

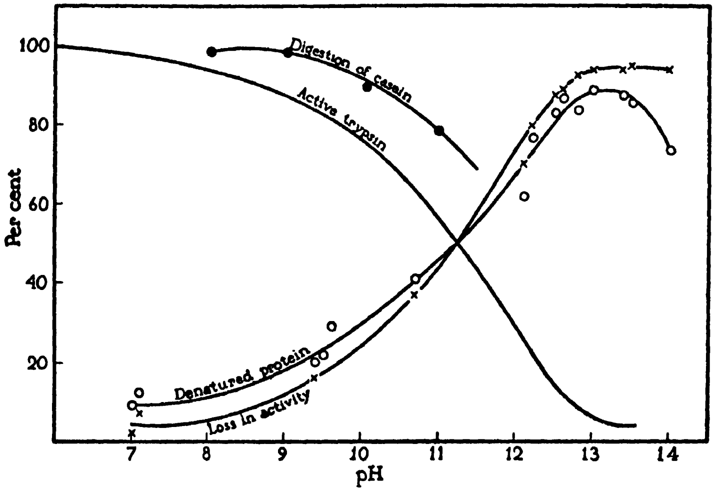

FIG. 2. Loss in activity and formation of reversibly denatured protein in trypsin solutions at various pH and 0°C.

basis to account for the subsequent decrease in the rate of digestion as the solution becomes increasingly alkaline. The effect of the pH on the ionization of the protein together with the present experiments, however, furnishes a complete picture of the pH activity curve of trypsin. In acid solutions trypsin is present in the active form but the substrate protein is almost entirely in the form of positive ions which are not digested by trypsin. As the alkalinity increases, the percentage of substrate protein present as negative ions increases, and therefore, the rate of digestion increases. As the alkalinity is increased still further the percentage of trypsin present in the active form begins to decrease. Since the rate of digestion is proportional to the product of the concentration of negative protein ions times the concentration of active trypsin there is a point at which this value and hence the rate of hydrolysis is a maximum and the position of this maximum will depend upon the substrate protein used.

2. *Effect of the Trypsin Concentration.*—The preceding experiments show that there is an equilibrium between active native and reversibly denatured trypsin which is shifted toward the denatured side as the alkalinity increases beyond pH 8.0 and towards the active side as the solution is made acid. In order to determine the nature of this equilibrium an experiment was carried out in which the concentration of trypsin was varied. A series of solutions of different concentrations of trypsin were cooled to 0°C. and sodium hydroxide added so that the final pH of all the solutions was 13.0. The solutions were then immediately analyzed for activity by the urea method (Anson and Mirsky (10 *b*)) and also by precipitation in 4/5 M sodium sulfate. Aliquot portions of the alkaline solutions were diluted with hydrochloric acid, the pH adjusted to 2.3 and the activity of these solutions determined. The latter method determines the "potential activity" of the solution since under these conditions the reversibly denatured trypsin reverts to the active native form. The results of the experiment are shown in Table I, in which the loss in activity has been expressed as per cent of the original activity. The samples taken into acid show practically no loss in activity. The loss in activity is, therefore, reversible. The table shows that the results by the urea method agree with those by the sodium sulfate method in concentrated solutions. In more dilute solution the sodium sulfate method cannot be

used since very low concentrations of denatured protein do not precipitate. The table also shows that the per cent denatured is independent of the original trypsin concentration. It follows, therefore, that the reaction—native trypsin protein to reversibly denatured trypsin protein—is of the same order in both directions and may be expressed by the relation

$$\frac{D}{A - D} = K_d$$

where A and D are the concentrations of potentially active and of reversibly denatured protein and $(A - D)$ is native active protein.

TABLE I

Denaturation of Various Concentrations of Trypsin pH 13.0 at 0°C.

Original concentration of active trypsin (active native protein), *mg. N/ml*	3.83	0.765	0.153	0.0306	0.0153
Per cent loss in original activity					
Urea method	97	96	94	91	87
Sodium sulfate method	95	92	(77)		
Per cent original activity recovered					
Hydrochloric acid solutions	98.5	96	98	99	95

K_d is the equilibrium constant of the reaction. Therefore, $D = \alpha A$ where

$$\alpha = \frac{K_d}{1 + K_d}$$

K_d is a function of the pH of the solution and becomes larger as the alkalinity is increased.

B. Irreversible Inactivation of Trypsin

The inactivation of trypsin described in the preceding experiments is due to the formation of reversibly denatured protein which is in equilibrium with the active native protein. If the solutions are allowed to stand, secondary changes occur and the activity can no longer be completely recovered. The velocity of these reactions and the nature of the products formed depend upon the temperature and the pH of the solution. The following experiments describe in detail the course of the reaction and the products formed under various conditions of pH and temperature. Equations have been derived which predict the course of the inactivation.

1. Inactivation at 0°C. and pH 13.0.—The results of an experiment in which trypsin solutions at pH 13.0 were allowed to stand at 0°C. are shown in Table II. There is no change in the total protein but about four-fifths of the total native protein is changed practically instantly to denatured protein. At the same time more than four-fifths of the activity disappears and hence a small amount of inactive native protein is probably formed.[1] The protein is now mostly in the form of reversibly denatured protein which changes rapidly to inactive protein and as a result the potentially active protein (active native protein present in the original solution plus reversibly denatured protein) decreases and has practically disappeared at the end of 15 minutes. At the same time the potential activity (activity present in the original solution plus activity recovered in acid solution) decreases in almost exact proportion so that the specific activity of the potentially active

TABLE II

Inactivation of Trypsin Solutions at pH 13.0 and 0°C.

Time at 0°C.	Total protein	Total native protein	Activity	Total denatured protein	Inactive protein	Potentially active protein	Potential activity	Specific activity of potentially active protein
min.	*N/ml. mg.*	*N/ml. mg.*	$[T.U.]^{Hb}_{ml.}$	*N/ml. mg.*	*N/ml. mg.*	*N/ml. mg.*	$[T.U.]^{Hb}_{ml}$	$[T.U.]^{Hb}_{mg.P.\ N}$
0 (control)	5.96	5.90	1.00	0	0	5.9	1.02	0.173
0.083	6.00	1.13	0.058	4.77	0.90	5.0	0.90	0.180
0.167	6.10	0.92	0.056	5.00	0.70	5.2	0.88	0.170
0.50		1.06	0.098	4.84	1.20	4.7	0.80	0.170
1.00	5.90	0.85	0.034	5.05	1.85	4.0	0.71	0.169
5.00		0.78	0.017	5.12	4.70	1.20	0.24	0.200
15.00	5.90	0.67	0.0012	5.23	5.80	0.10	0.025	(0.25)

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[1] This inactive native protein could not be determined by direct analysis in this experiment since the solution was too dilute.

protein remains constant. The potentially active protein is determined after heating the solution in dilute acid. This procedure denatures and removes the inactive native protein, referred to above. The constant activity of the potentially active protein fraction shows that any irreversible loss in activity under these conditions is accompanied by a corresponding loss in either active native or reversibly denatured protein.

2. *Inactivation at pH 13.0 and 30°C.*—If the preceding experiment is performed at 30°C. instead of at 0°C. the total denatured protein is changed to inactive protein very rapidly so that the potential activity drops to practically zero during the first few minutes while the potentially active protein drops to about 8 per cent of its original value (Table III). There is evidently a small quantity of protein which appears in the potentially active protein fraction but which is inactive. This protein (or a similar one) is also found in crude trypsin preparations and cannot be separated from the trypsin protein by heat denaturation since it also reverts to the native form on cooling. It can be separated from the active protein by repeated fractionation but so far it has not been possible to find any quantitative method of separation. On longer standing at pH 13.0 the total protein begins to decrease and at the same time there is an increase in the amino nitrogen content of the solution due to hydrolysis of the inactive protein.

TABLE III

Inactivation of Trypsin pH 13.6 (in N/1 Sodium Hydroxide) at 30°C. Solutions Acidified after Various Times. Total Nitrogen = 7.35 Mg./Ml.

Time at 30°C.	Potentially active protein	Amino nitrogen	Non-protein nitrogen	Total protein	Potential activity
	N/ml. mg.	*N/ml. mg.*	*N/ml. mg.*	*N/ml. mg.*	$[T.U.]^{Hb}_{ml.}$
0 (acid control)	6.35	0.70	1.0	6.35	1.08
0 pH 13.6	0.65		1.8	5.55	0.051
5 min.	0.45		2.2	5.15	0
15 "	0.45	0.76	2.6	4.75	0
1.5 hrs.	0.55	0.90	3.6	3.75	0
3.5 "		1.00			
5.5 "		1.06	5.3	2.05	0
16.0 "	0.45	1.40	5.6	1.75	0

Kinetics of the Formation of Inactive Protein at pH 13.0.—The preceding experiments have shown that at pH 13.0 there is an instantly reversible equilibrium between active native and reversibly denatured protein and that on further standing the reversibly denatured protein is gradually changed to inactive protein. Since $D = \alpha A$ (*cf.* p. 598) the reaction may be considered to go directly from native to inactive protein and the equation for the velocity of the reaction may be written for dilute trypsin solutions

$$-\frac{dA}{dt} = K_1 A$$

whence $K_1 = \frac{2.3}{t} \log \frac{A_o}{A}$ in which A is the potential activity at the time t. This may be written

$$K_1 = \frac{2.3}{t} \log \frac{1}{f_A}$$

where $f_A = \frac{A}{A_o}$; *i.e.*, the fraction of the total original activity present at the time t. The results of an experiment at pH 12.8 and 30°C. have been plotted in Fig. 3 where the fraction of the activity present has been plotted against the time. The ln of $\frac{1}{f_A}$ has also been plotted against the time and the resulting curve is a straight line showing that the equation agrees with the experimental results. The value of the velocity constant is 0.364 per minute. The loss in activity in this experiment is less rapid than in the experiment reported in Table III in which much more concentrated trypsin solution was used. Theoretically the per cent loss should be independent of the concentration and this is true below a concentration of about 1.0 mg. protein nitrogen/ml. As the concentration is increased above this the reaction apparently proceeds more rapidly. This effect may be due to the difficulty of mixing the very concentrated alkali with the trypsin solution.

3. Inactivation in Acid Solution at 30°C.—On the acid side of pH 2.0 the inactivation of trypsin is quite similar to that just described in alkaline solution. The result of an experiment under these conditions is shown in Table IV. There is no decrease in the total protein. The potential activity decreases slowly and there is a corresponding

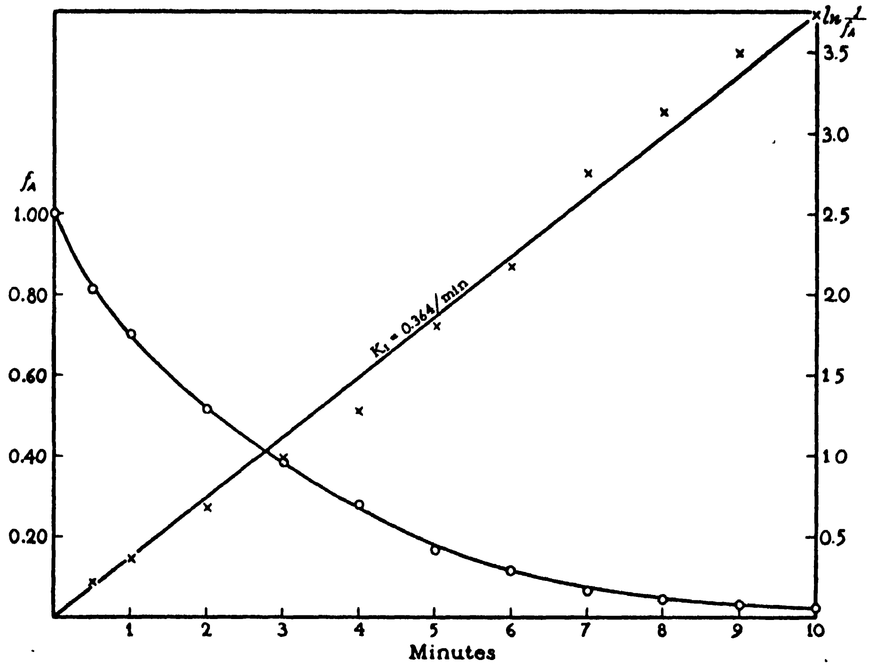

FIG. 3. Decrease in activity of trypsin solutions at 30°C. and pH 12.8

TABLE IV

Inactivation of Trypsin in M/2 Hydrochloric Acid at 30°C. Total Nitrogen = 1.15 Mg./Ml. Protein Nitrogen = 0.80 Mg./Ml.

Time	Total protein	Potentially active protein	Potential activity	Specific activity
hrs.	*N/ml.* *mg.*	*N/ml.* *mg.*	$\left[T.U.\right]^{Hb}_{ml.}$	$\left[T.U.\right]^{Hb}_{mg.\ P.N}$
0	0.80	0.65	0.107	0.165
1	"	0.58	0.0940	0.162
3	"	0.49	0.0730	0.149
5	"	0.49	0.0680	0.139
8	"	0.47	0.0650	0.138
12	"	0.40	0.0493	0.123
16	"	0.38	0.0442	0.126
20	"	0.35	0.0340	0.097
24	"	0.30	0.0255	0.085
32	"	0.30	0.0204	0.068
48	"	0.26	0.0068	0.026

decrease in potentially active protein so that its specific activity is nearly constant. There is again a small amount of protein which appears in the potentially active protein fraction but which is not active as in the case of the alkaline inactivation. The mechanism of the reaction consists in the formation of inactive protein from active native protein and may be represented by the same equation as that derived for the inactivation in alkaline solution. In acid solution no denatured protein can be detected but in any case the formulation of the reaction is the same. The results of an experiment in M/2 hydrochloric acid at 30°C. are shown in Fig. 4. The fraction of the activity remaining and the ln of the reciprocal of this value have been plotted against the time in hours. The logarithmic plot is a straight line showing again that the equation agrees with the experimental results. The value of the inactivation constant K_1 in this case is 0.001 per minute at 30°C. which is less than 1/300 of that at pH 13.0.

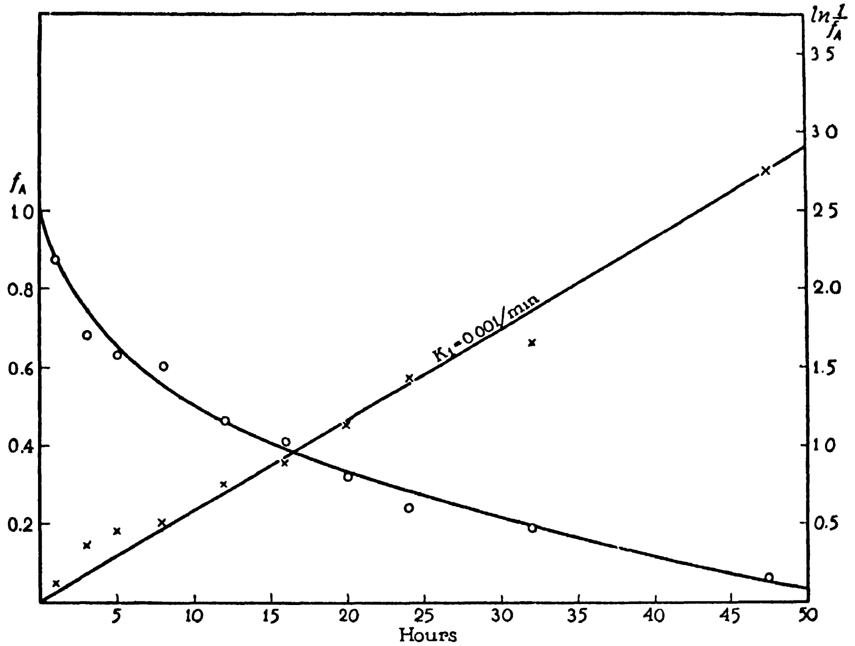

FIG. 4. Loss in activity of trypsin solutions in M/2 hydrochloric acid at 30°C.

4. *Inactivation of Trypsin from pH 2.5 to pH 10.0 at 30°C.*—From pH 2.0 to 10.0 the inactivation of trypsin at 30°C. is accompanied by

hydrolysis of the protein and the appearance of non-protein compounds. No denatured protein can be detected in the solution. The results of an experiment at pH 8.0 and 30°C. are shown in Table V. The decrease in potential activity is accompanied by a corresponding decrease in total protein and an increase in non-protein nitrogen. In more strongly alkaline solutions there is an equilibrium between active native and reversibly denatured protein and it follows, theoretically, that there must be some reversibly denatured protein present in the solution at any pH although it may not be in sufficient quantity to detect experimentally. It seems probable, therefore, that the inactivation is due to digestion of the reversibly denatured protein by the remaining active native protein. This assumption is confirmed by the effect of temperature upon the rate of inactivation. The temperature coefficient is higher than that usually found for the velocity of digestion and agrees approximately with the effect of temperature upon the formation of reversibly denatured trypsin (3). According to this mechanism the inactivation of trypsin in this range may be expressed by the following reactions.

TABLE V

Inactivation of Trypsin at 30°C. in M/10 Borate Buffer, pH 8.0

Time	Non-protein nitrogen	Total protein	Potential activity	Specific activity
hrs.	*N/ml. mg.*	*N/ml. mg.*	$[T.U.]^{Hb}_{ml.}$	$[T.U.]^{Hb}_{mg. P.N}$
0	0.30	0.85	0.145	0.170
0.01	0.34	0.81	0.141	0.170
0.1	0.40	0.75	0.134	0.179
0.25	0.48	0.67	0.116	0.173
0.5	0.58	0.57	0.105	0.184
0.75	0.62	0.53	0.088	0.166
1.0	0.63	0.52	0.084	0.161
1.5	0.68	0.47	0.075	0.160
2.0	0.69	0.46	0.075	0.163
3.0	0.71	0.44	0.066	0.150
4.0	0.75	0.40	0.058	0.145
5.0	0.76	0.39	0.056	0.144
6.0	0.77	0.38	0.051	0.139
23.5	0.86	0.29	0.029	0.100

1. Active native trypsin ⇄ reversibly denatured trypsin.
2. Denatured trypsin + active native trypsin → non-protein nitrogen + active native trypsin (catalytic reaction).

The first reaction is instantaneous while the second is a time reaction. The final rate of the reaction depends first, on the degree of denaturation which is a function of pH and temperature (2 *b*, 3), and second, on the velocity constant which expresses the rate of digestion of the denatured trypsin by the active native trypsin. If it be assumed that the rate of digestion is proportional to the concentration of the active native trypsin as well as to the concentration of reversibly denatured protein D, then the rate of hydrolysis of trypsin is

$$-\frac{dA}{dt} = K_e D (A - D)$$

where A equals the concentration of potentially active trypsin (active native plus reversibly denatured).

Substituting for D its equivalent $D = \alpha A$ (p. 598) the differential equation becomes

$$-\frac{dA}{dt} = K_e \alpha (1 - \alpha) A^2 = K_2 A^2$$

which on intergration gives

$$\frac{A_0}{A_0 A} - \frac{A}{A_0 A} = K_2 t \tag{2}$$

or:

$$\frac{1}{f_A} - 1 = K_2 A_0 t \qquad \text{where } f_A = \frac{A}{A_0} \tag{2 a}$$

When f_A is plotted against t the resulting curve is a hyperbola assymptotic to the t axis and intercepting the f_A axis at $f_A = 1$.

This equation, which is of the bimolecular type, was found to fit the experimental data for the inactivation of dilute solutions of trypsin in the range of pH 4.0 to 10.0 to 30°C. Buffer solutions kept the pH constant during the inactivation period.

Table VI shows the application of the bimolecular equation (2 *a*) to several cases.

Fig. 5 shows the plotted data for pH 9.0. The smooth curve was drawn through calculated values by substituting the average value of $K_2\ A_o$ = 2.66/hour. For comparison the values for ln $\frac{1}{f_A}$ were plotted against time. The logarithmic curve shows clearly that the inactivation of trypsin at this pH is not a monomolecular process.

TABLE VI

Rate of Inactivation of Dilute Trypsin Solution in M/10 Borate or Phosphate Buffer Solutions of Various pH at 30°C. Initial Activity A_o = 0.0051 $[T.\ U.]^{Hb}_{ml.}$. Protein Nitrogen = 0.03 Mg./Ml.

pH 9.6			pH 9.0			pH 7.0		
t	f_A	$K_2A_0 = \frac{1/f_A - 1}{t}$	t	f_A	K_2A_0	t	f_A	K_2A_0
min.			*hrs.*			*hrs.*		
5	0.76	0.064	0.1	0.82	2.20	0.25	0.93	0.28
10	0.66	0.052	0.2	0.63	1.95	0.50	0.83	0.40
20	0.53	0.045	0.3	0.52	3.07	0.75	0.81	0.31
40	0.37	0.043	0.4	0.47	2.81	1.00	0.76	0.32
60	0.25	0.050	0.5	0.45	2.44	2.00	0.64	0.28
90	0.145	0.065	0.75	0.35	2.48	4.00	0.50	0.25
120	0.12	0.061	1.00	0.27	2.70	7.00	0.37	0.24
180	0.08	0.064	2.00	0.16	2.62	13.00	0.25	0.23
240	0.06	0.065	3.00	0.10	3.00	23.00	0.15	0.25
Average 0.057/min.			Average 2.66/hr.			Average 0.26/hr.		
$K_2 = \frac{0.057}{0.03}$ = 1.9/min. per mg. protein nitrogen/ml.			$K_2 = \frac{2.66}{60 \times 0.03}$ = 1.5/min. per mg. protein nitrogen/ml.			K_2 = 0.16/min. per mg. protein nitrogen/ml.		

f_A = fraction of potential activity (original activity plus activity recovered in acid solution)

5. Inactivation of Trypsin from pH 10.0 to 12.0.—From pH 2.5 to 10.0 the inactivation of trypsin is due to destruction of protein and the appearance of non-protein nitrogen while on the alkaline side of pH 12.0 the inactivation is due to the formation of inactive from active protein. The first reaction is bimolecular, the second monomolecular. In the intermediate range of pH between 10.0 and 12.0 part of the protein is digested and appears as non-protein nitrogen and part of it is transformed to inactive protein. The pH at which the formation of inactive protein becomes significant depends on the temperature. As the temperature is lowered the rate of digestion decreases more than the rate of formation of inactive protein and inactive protein accumulates in the solution; thus, at pH 9.0 and 30°C. no inactive protein

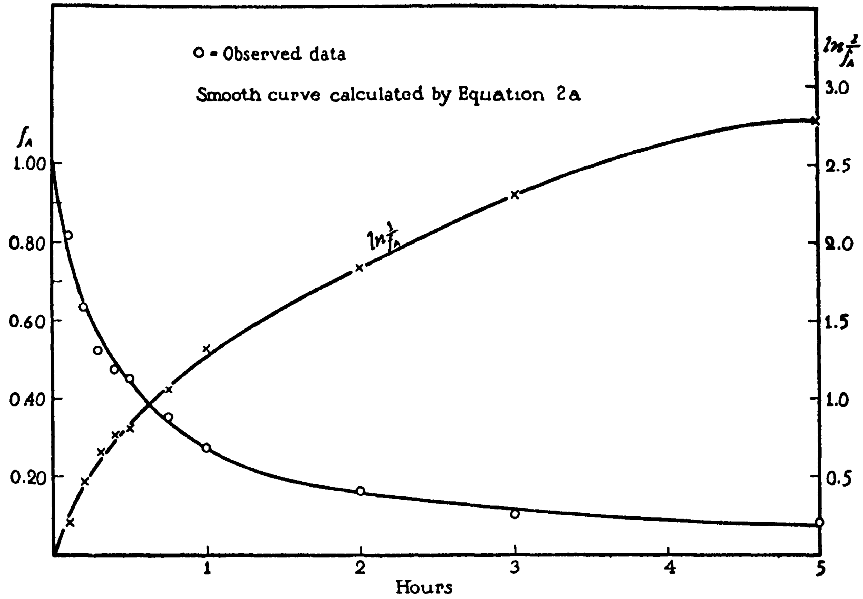

FIG. 5. Calculated and observed loss in activity of trypsin solutions in M/10 borate buffer pH 9.0 and 30°C.

TABLE VII

Inactivation of Trypsin pH 9.0 at 0°C. Total Nitrogen = 6.90 Mg./Ml.

Time	Non-protein nitrogen	Total protein	Inactive protein	Potentially active protein	Potential activity	Specific activity
min.	*N/ml. mg.*	*N/ml. mg.*	*N/ml. mg.*	*N/ml. mg.*	$[T.U.]^{Hb}_{ml.}$	$[T.U.]^{Hb}_{mg. P. N}$
0(control)	0.80	6.10	0	6.10	1.04	0.170
0.083	0.85	6.05	0.45	5.60	0.97	0.173
0.167	0.90	6.00	1.00	5.00	0.87	0.170
0.500	1.00	5.90	1.70	4.20	0.71	0.169
1.00	1.20	5.70	2.50	3.20	0.53	0.171
5.00	2.20	4.70	2.40	2.30	0.39	0.170
15.00	4.05	2.85	0.50		0.39	

appears in the solution and the reaction is purely bimolecular, while at 0°C. inactive protein accumulates in the solution and the equation for the reaction is the sum of a mono- and bimolecular equation. The high temperature coefficient of the digestion reaction is due to the fact that the velocity of this reaction is determined by the equilibrium between active native and reversibly denatured protein. This equilibrium constant has a high temperature coefficient (3) and the equilibrium is shifted to the denatured form as the temperature is increased. The results of an experiment done at pH 9.0 and 0°C. are shown in Table VII. The total protein decreases rapidly and increasing quantities of non-protein nitrogen and inactive protein are formed. The potentially active protein, therefore, decreases more rapidly than the total protein. The specific activity of the potentially active protein remains constant. The mechanism of the reaction consists in the formation of inactive protein (as discussed under No. 1) which is a monomolecular reaction and the digestion of denatured protein (discussed under No. 4) which is a bimolecular reaction. The inactive protein is also subsequently hydrolyzed and its concentration, therefore, goes through a maximum. This is a secondary reaction and does not affect the kinetics of the inactivation of the original trypsin solution. The entire process, therefore, may be represented by the following differential equations.

1. Active native trypsin → inactive protein (monomolecular); *i.e.*,

$$-\frac{dA}{dt} = K_1 A$$

2. Active native trypsin $\rightleftharpoons$ reversibly denatured trypsin and active trypsin + denatured trypsin = non-protein nitrogen + active trypsin, (bimolecular); *i.e.*,

$$-\frac{dA}{dt} = K_2 A^2$$

The rate of the complete process then is the sum of the rates of both reactions

$$-\frac{dA}{dt} = K_1 A + K_2 A^2 \quad \text{or} -\frac{df_A}{dt} = K_1 f_A + K_2 A_o f_A^2$$

which on integration becomes

$$2.3 \log \frac{K_1 + K_2 A_o f_A}{(K_1 + K_2 A_o) f_A} = K_1 t \tag{3}$$

The values for K_1 and K_2 can be obtained from two points. Approximate values for K_1 and K_2 can be obtained as follows:
The differential equation

$$-\frac{d f_A}{d t} = K_1 f_A + K_2 A_o f_A^2$$

can be written as

$$-\frac{d \ln f_A}{d t} = K_1 + K_2 A_o f_A$$

Hence

$$-\frac{d \ln f_A}{d t} = K_1 + K_2 A_o \qquad \text{as } f_A \text{ approaches } 1.0$$

and

$$-\frac{d \ln f_A}{d t} = K_1 \qquad \text{as } f_A \text{ approaches } 0.$$

Thus, if the values of $\ln 1/f_A$ are plotted against the time, the slope of the curve at $t = 0$ is $K_1 + K_2 A_o$, and the slope at $t = \alpha$ is K_1.

The reaction is of this type at 30°C. even at pH 12.0. The results of an experiment at pH 11.7 and 30°C. calculated in this way are shown in Table VIII and are plotted in Fig. 6. The calculated values for t agree very well with the observed values.

Effect of the Concentration of the Enzyme Solution.—The preceding experiments were carried out with dilute trypsin solutions and under these conditions the equation agrees very well with the experimental results. However, when more concentrated solutions were used the agreement was not satisfactory. The calculated bimolecular constant, K_2, decreases as the process proceeds indicating that something happens which decreases the rate of digestion. A similar phenomenon is well known in relation to the kinetics of the hydrolysis of ordinary proteins by trypsin. In this case the reaction should be monomolecular but actually the monomolecular constant decreases quite rapidly with time. This anomaly can be quantitatively explained by assuming that the products of digestion form an easily dissociated compound with the active enzyme; *i.e.*, enzyme plus products equals enzyme-products. The complex of enzyme and products is inactive but the

TABLE VIII

Inactivation of Trypsin pH 11.7 at 30°C. Initial Activity = 0.0054 $[T.U.]^{Hb}_{ml.}$
Application of Equation 3

$$t = \frac{1}{K_1} \ln \frac{K_1 + K_2 A_o f_A}{(K_1 + K_2 A_o) f_A} \qquad K_1 = 0.012 \qquad K_2 A_o = 0.04$$

Time		f_A	K unimolecular Equation 1	K bimolecular Equation 2
Observed	Calculated			
min.	*min.*			
5	4.1	0.84	0.035	1.2
8	10.0	0.65	0.054	2.1
10	12.0	0.60	0.051	2.1
15	14.0	0.52	0.043	1.9
20	20.0	0.45	0.040	1.9
25	24.0	0.40	0.037	1.9
60	75.0	0.137	0.033	3.1
120	125.0	0.062	0.023	3.9

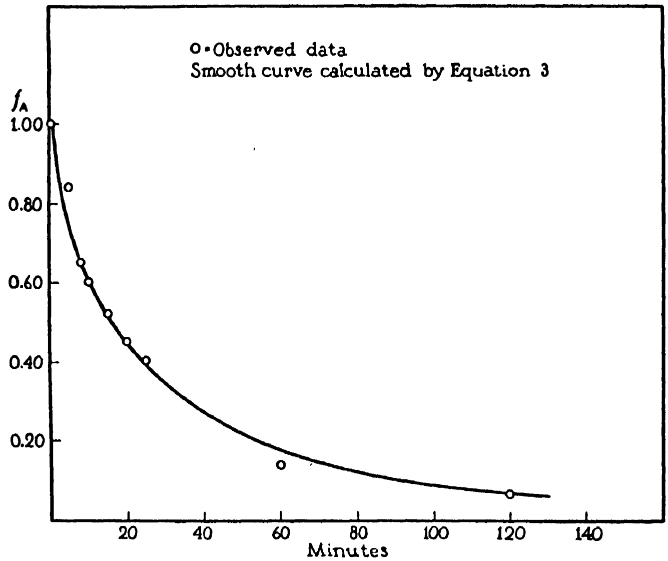

FIG. 6. Calculated and observed values for the loss in activity of trypsin at pH 11.7 and 30°C.

per cent of the enzyme present in this form depends upon the concentration so that when a solution is diluted the compound dissociates and the enzyme again becomes active. This mechanism has been checked experimentally for both the digestion of proteins by trypsin (7) and for the inactivation of trypsin (8) by the addition of the products of digestion to the solution. Evidently such a mechanism would account qualitatively for the results observed in the present case since it would predict that the rate of digestion would become slower as the process continued and also that the effect would be more noticeable in concentrated than in dilute solution. If this mechanism is taken into account the inactivation of more concentrated trypsin solutions may be satisfactorily calculated. The equation derived, however, contains two arbitrary constants so that the agreement with the observed data is not very convincing. The derivation is somewhat long and complicated and does not appear to be of sufficient interest to report in detail.

Experimental Methods

A. Material

The material used was crystalline trypsin prepared from beef pancreas, as previously described (2 *a*). The trypsin was freed from ammonium salts either by washing the crystalline filter cake with a saturated solution of magnesium sulfate or by dialysis at 5°C. of a solution of trypsin against N/200 hydrochloric acid. The methods of analysis described apply only to such a solution of purified trypsin. They cannot be applied to solutions containing ammonium salts nor to solutions of crude trypsin containing large amounts of inactive protein.

B. Methods of Analysis

All values for protein materials are expressed in terms of their nitrogen content.

1. Total Protein Nitrogen.—5 ml. of solution added to 5 ml. 5 per cent trichloracetic acid and the suspension heated for 5 minutes at 75°C. and cooled. The suspension is filtered on hardened paper and the precipitate washed with 2.5 per cent trichloracetic acid. The precipitate is then washed with water into a Kjeldahl flask and the total nitrogen determined (9). The supernatant solution may be analyzed for nitrogen and the protein nitrogen calculated by difference from this figure and the total nitrogen content of the solution.

2. Total Denatured and Total Native Protein.—1 ml. solution added to 4 ml. M/1 sodium chloride[2] and centrifuged.

Precipitate.................................*Total denatured protein.*
Supernatant...............................*Total native protein.*

3. Potentially Active Protein and Inactive Protein.—Solution diluted to < 0.05 M salt concentration, titrated to pH 2.0, heated to 80°C. for 1 minute, cooled to 20°C. 1 ml. 5 M sodium chloride added to 5 ml. of solution and centrifuged.

TABLE IX

Denaturation of Trypsin pH 13.0 in N/2 Sodium Hydroxide at 0°C. Total Nitrogen = 7.3 Mg./Ml. 1 Ml. Added to 4 Ml. of Various Salt Solutions

Salt solution	Sodium chloride				Sodium sulfate						Magnesium sulfate
Concentration...........	2.5 M	2.0 M	1.5 M	1.0 M	M/1	3/4 M	M/2	M/4	M/8	M/16	1/2 saturated
					Precipitate in all						
Total N in filtrate, *mg*..............	2.3	2.3	2.6	3.8	2.3	2.1	2.6	4.1	5.8	6.9	2.4
N in precipitate by difference, *mg*....	5.0	5.0	4.7	3.5	5.0	5.2	4.7	3.2	1.5	0.4	4.9

Precipitate...........*Inactive protein.*
= *Irreversibly denatured plus inactive native protein.*
Supernatant..........*Potentially active protein.*
= *Active native plus reversibly denatured.*

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[2] The value obtained for the total denatured and total native fraction by the method outlined is independent of the nature and concentration of the salt solution used over quite a wide range. This is shown in Table IX which summarizes the result of an experiment in which the denatured protein was precipitated by sodium chloride, sodium sulfate or magnesium sulfate. As the table shows, the quantity of denatured protein precipitated was the same for all three salts in the higher concentrations. The solutions were also analyzed by means of activity determinations as summarized in Table IV.

Activity Determinations

The activity was determined by the hemoglobin method (10 *b*). Commercial hemoglobin was used. The specific activity of crystalline trypsin was found to be 0.17 $[\text{T. U.}]^{\text{Hb}}_{\text{mg. P. N.}}$

The results were checked occasionally by other methods such as casein digestion or gelatin viscosity (11). The per cent inactivation as determined by the various methods agrees, as shown in Table X.

TABLE X

Comparison of Various Methods of Measuring Loss in Activity of Trypsin Solution pH 6.8 on Standing at 30°C.

Method	Digestion of casein	Viscosity of gelatin	Hemoglobin
Per cent activity lost after 0.5 hr.	34	37	40
1.0 "	48	48	46
2.0 hrs.	52	53	55
4.0 "	63	58	58
5.5 "	69	69	66

Methods

1. Activity of the Solution.—

(*a*) *Urea Method.*—1 ml. of the solution added to urea solution and the activity determined as described by Anson and Mirsky (10 *a*).

(*b*) *Precipitation.*—1 ml. of solution precipitated with salt as for total denatured protein and activity determined on the supernatant fluid.

The results of these two methods agree, as Anson and Mirsky have shown, and as has been confirmed during the course of this work.

2. Potential Activity.—Solution diluted to < 0.05 M salt concentration, titrated to pH 2.0, kept at 20°C. for 1 hour and activity determined.

Most of the analytical determinations reported in this paper were done by Miss Margaret McDonald.

SUMMARY

1. The rate of inactivation of crystalline trypsin solutions and the nature of the products formed during the inactivation at various pH at temperatures below 37°C. have been studied.

2. The inactivation may be reversible or irreversible. Reversible inactivation is accompanied by the formation of reversibly denatured protein. This denatured protein exists in equilibrium with the native active protein and the equilibrium is shifted towards the denatured form by raising the temperature or by increasing the alkalinity. The decrease in the fraction of active enzyme present (due to the formation of this reversibly denatured protein) as the pH is increased from 8.0 to 12.0 accounts for the decrease in the rate of digestion of proteins by trypsin in this range of pH.

3. The loss of activity at high temperatures or in alkaline solutions, just described, is very rapid and is completely reversible for a short time only. If the solutions are allowed to stand the loss in activity becomes gradually irreversible and is accompanied by the appearance of various reaction products the nature of which depends upon the temperature and pH of the solution.

4. On the acid side of pH 2.0 the trypsin protein is changed to an inactive form which is irreversibly denatured by heat. The course of the reaction in this range is monomolecular and its velocity increases as the acidity increases.

5. From pH 2.0 to 9.0 trypsin protein is slowly hydrolyzed. The course of the inactivation in this range of pH is bimolecular and its velocity increases as the alkalinity increases to pH 10.0 and then decreases. As a result of these two reactions there is a point of maximum stability at about pH 2.3.

6. On the alkaline side of pH 13.0 the reaction is similar to that in strong acid solution and consists in the formation of inactive protein. The course of the reaction is monomolecular and the velocity increases with increasing alkalinity. From pH 9.0 to 12.0 some hydrolysis takes place and some inactive protein is formed and the course of the reaction is represented by the sum of a bi- and monomolecular reaction. The rate of hydrolysis decreases as the solution becomes more alkaline than pH 10.0 while the rate of formation of inactive protein increases so that there is a second point at about pH 13.0 at which the rate of inactivation is a minimum. In general the decrease in activity under all these conditions is proportional to the decrease in the concentration of the trypsin protein. Equations have been derived which agree quantitatively with the various inactivation experiments.

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ON NORRIS' THEORY FOR THE SHAPE OF THE MAMMALIAN ERYTHROCYTE

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Norris in 1882, Rice in 1914, Gough in 1924, and several others, have commented on the similarity between the shape of the mammalian red cell and that of the "myelin forms" which are assumed by droplets of lecithin in water. These latter are described as circular discs, dumb-bell-shaped in cross-section, and varying in diameter from 5μ to 10μ. They are apparently produced by physical forces at work at the interfaces between the lecithin droplets and the fluid surrounding them and Norris, Rice, Gough, and others have suggested that the biconcave shape of the mammalian red cell is brought about in a similar way. Thus, as Gough puts it, there are two sets of forces operating, the first of which tends to produce contraction of the surface and the spherical form, and the second of which tends to bring about expansion of the surface and a very flattened form: balanced against each other these two sets of forces maintain the typical discoidal form.

At one time (1926) this idea was very attractive to me, and with the help of Professor C. G. Darwin I tried to put it into mathematical form. The result was, and is, disappointing, but it is suggestive, and so I have decided to publish it, not because it means very much as it stands, but in the hope that by an elaboration or modification of the idea Norris' theory may ultimately be expressed in quantitative terms.

I

The red cell, being invested with a membrane and containing fluid inside, is presumably in hydrostatic equilibrium; *i.e.*,

$$P = T(1/\rho_1 + 1/\rho_2) \tag{1}$$

where P is the net pressure acting on a component of membrane which has tension T, and where ρ_1 and ρ_2 are the principal radii of curvature of the element. It is clear that if the tension is uniform we shall need to suppose different pressures applied at different points along the membrane if we are to get the required discoidal form. These could be evaluated by finding ρ_1 and ρ_2 graphically from a drawing of a cross-sectional view of the cell, but it simplifies matters considerably if we replace this cross-sectional view by a figure constructed in the following way.

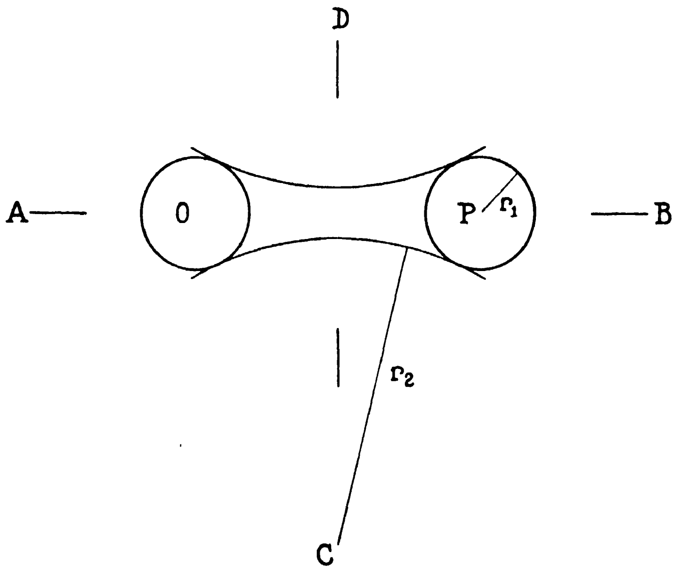

FIG. 1. To illustrate the method of approximately outlining the red cell in cross-section, as explained in the text.

Draw a line *AB* whose length (conveniently 10 scale units) represents the diameter of the cell. With centers *O*, *P*, draw two circles whose radii $= r_1 = OA = OB$ are each half the greatest thickness of the cell. With centers on a line *CD*, bisecting *AB*, draw two other circles which touch the first two without cutting them and which at the same time pass through the points on *CD* corresponding to the least thickness of the cell. Let the radii of these latter circles be r_2. The figure bounded by the arcs of the smaller circles outside the points of contact and by the arcs of the larger circles inside the points of contact then very closely approximates to the form of the cross-section of the cell, at least in the case of all animals for which we have measurements (see

Fig. 1). The form of the figure, moreover, is determined by only three measurements, *viz.* the diameter, the greatest thickness, and the least thickness, and if we have $r_2/r_1 = k$, the value of this constant is approximately 5.3 for the cells of man, 7.9 for the cells of the rabbit, and 3.1 for the cells of the sheep (see Ponder, 1930). If this approximation to the cross-section revolves about *CD*, a solid of revolution which approximates to the erythrocyte is formed, and it is unnecessary to point out that not only can ρ_1 and ρ_2 be easily found for all points on the surface, but that the volume and area of the resulting solid of revolution are easily calculable.

Taking the erythrocyte of man drawn on such a scale that the semidiameter = 10 units, and evaluating the curvatures, we get the following figures, which, of course, are to be regarded as approximations only.

Units	ρ_1	ρ_2	P
0–6.1	−15.2	−15.2	−0.13
6.1		Point of inflexion	
7.0	+2.8		+0.36
8.0	+2.8	+28.2	+0.40
9.0	+2.8	+14.2	+0.43
10.0	+2.8	+10.0	+0 46

Plotting the values of P, we get Fig. 2, Curve *a*, which, although it does not show the values of P accurately, shows them to the same degree of approximation as the above system of circles provides us with a figure of the shape of the cell. In the same figure, Curve *b*, I have dotted in what is probably more nearly the correct curve. It thus appears that we would have to imagine a pressure directed outwards over the equatorial regions of the cell, and a smaller pressure directed inwards over the biconcavities, if we were to account for the biconcave shape in this way.

The idea that such pressures really exist is, of course, untenable, but the "outward pressure over the equatorial regions" is the same as Gough's idea of an expansive force (which Gough even went the length of attributing to an oriented repulsion of the pigment molecules in the cell interior). The reader will easily grasp the similarity if he imagines a spherical balloon subjected to an outward pressure along the equator. The volume must remain constant, and so the extension of area is accompanied by the sphere becoming somewhat like a spheroid, and the more eccentric this spheroid is, the greater is the area/volume ratio. But, even when this eccentricity is very great we can still get a greater extension of area for the same volume by allowing the flattish surfaces of the spheroid to become biconcave; in fact, we can get almost any area/volume ratio we like; *i.e.*, the form of the body can be so adjusted as to allow for almost any degree of expansion of the area without any alteration of volume. But in so manipulating the form, we have to remember the condition for hydrostatic equilibrium, and it is clear that the best form will be one which has the desired area/volume ratio and which has at the same time a minimal value for

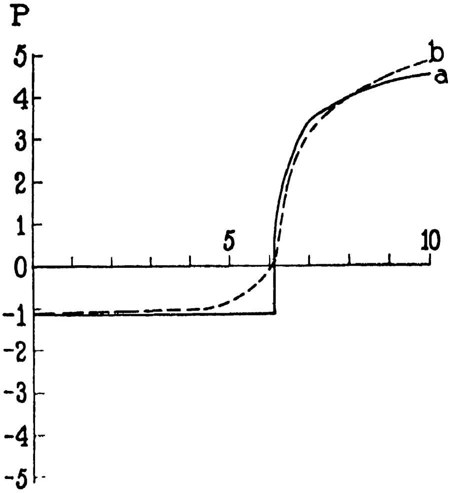

FIG. 2. Ordinate, arbitrary pressures acting normally to the membrane: abscissa, unit distances along the semidiameter. The full curve shows the figures given in the table of the text: the dotted curve gives the values obtained by an analysis of the curve which represents the actual cross-section of the cell.

$$\int (1/\rho_1 + 1/\rho_2)\, d\rho \qquad (2)$$

Let us see what happens when we try to put these ideas into mathematical form.

II

We require

$$\int \pi y^2 \, dx = \text{constant}$$

$$\int 2\pi y\sqrt{1 + \dot{y}^2} = \text{constant}$$

$$\text{and} \quad \int (1/\rho_1 + 1/\rho_2)\, d\rho \quad \text{a minimum}$$

Converting ρ_1 and ρ_2 to Cartesians, and varying y,

$$\delta \int \left(\frac{y\ddot{y}}{1 + \dot{y}^2} + \lambda y^2 + \mu y \sqrt{1 + \dot{y}^2}\right) dx$$

$$= \int \left(\frac{\ddot{y}\delta y + y\delta\ddot{y}}{1 + \dot{y}^2} - \frac{y\ddot{y}2\dot{y}\delta\dot{y}}{(1 + \dot{y}^2)^2} + 2\,\lambda y\delta y + \mu\delta y \sqrt{1 + \dot{y}^2} + \frac{\mu y\dot{y}\delta\dot{y}}{\sqrt{1 + \dot{y}^2}}\right) dx$$

$$= \left[\frac{y\delta\dot{y}}{1 + \dot{y}^2} - \frac{d}{dx}\left(\frac{y}{1 + \dot{y}^2}\right)\delta y - \frac{2y\dot{y}\ddot{y}}{(1 + \dot{y}^2)^2} \cdot \delta y + \frac{\mu y\dot{y}\delta y}{\sqrt{1 + \dot{y}^2}}\right]_0^1$$

$$+ \int \delta y \left\{\frac{d^2}{dx^2}\left(\frac{y}{1 + \dot{y}^2}\right) + \frac{\ddot{y}}{1 + \dot{y}^2} + \frac{d}{dx}\left(\frac{2y\dot{y}\ddot{y}}{(1 + \dot{y}^2)^2}\right) + 2\lambda y + \mu \sqrt{1 + \dot{y}^2}\right.$$

$$\left. - \mu \frac{d}{dx}\left(\frac{y\dot{y}}{\sqrt{1 + \dot{y}^2}}\right)\right\} = 0$$

The differential equation is

$$\frac{d}{dx}\left\{\frac{\dot{y}}{1 + \dot{y}^2} = \frac{2y\dot{y}\ddot{y}}{(1 + \dot{y}^2)^2}\right\} + \frac{\ddot{y}}{1 + \dot{y}^2} + \frac{d}{dx}\left(\frac{2y\dot{y}\ddot{y}}{(1 + \dot{y}^2)^2}\right) + 2\lambda y$$

$$+ \mu \sqrt{1 + \dot{y}^2} - \mu \frac{d}{dx}\left(\frac{y\dot{y}}{\sqrt{1 + \dot{y}^2}}\right) = 0$$

If $y = 0$ when $x = x_0, x_1$ the boundary terms go out, and the differential equation becomes

$$\frac{2\ddot{y}}{(1 + \dot{y}^2)^2} + 2\lambda y + \frac{\mu}{\sqrt{1 + \dot{y}^2}} - \frac{\mu y\ddot{y}}{(1 + \dot{y}^2)^{3/2}} = 0$$

Putting $1 + \dot{y}^2 = \omega^{-2}$, and $\omega = u.y$, successively, this reduces to

$$\omega^2 - 2\mu\omega y - \lambda y^2 = \text{a constant}$$

Call the constant θ; then

$$\lambda y^2 (1 + \dot{y}^2) + \mu y \sqrt{1 + \dot{y}^2} = \theta(1 + \dot{y}^2) + 1$$

This has to be solved to give the required form. If at $x = x_0, x_1, y = 0, \dot{y} = x$, then $\theta = 0$ and the solution is a sphere. The expression thus verifies. If $\dot{y}$ is finite at $x = x_0, x_1$ call it $\pm\alpha$. Then we have a body with points on it. Proceeding with this case, $\alpha^2 = -1 - 1/\theta$. For $\alpha^2 > 0$, $\theta < 0$ and $-1/\theta > 1$, so θ lies between 0 and -1.

If $\dot{y}$ is real when y is real, $y^2 > \theta/(\mu^2 + \lambda)$; *i.e.*, for a real form we require $\mu^2 > \lambda$. Further, when $\dot{y} = x$, $y^2 = \theta/\lambda$, and for this to have real roots λ must be negative for θ is negative.

The values of the constant being thus limited, the differential equation may be solved in the following way. Arbitrary values within the proper limits, are given to μ, λ, and θ. Values of $\dot{y}$ are then inserted in the differential equation and y solved for. We can then tabulate $\dot{y} = f(y)$ and $\dot{x} = f(y)$ and can plot values of $\dot{x}$ against y. From this plotted curve we then obtain

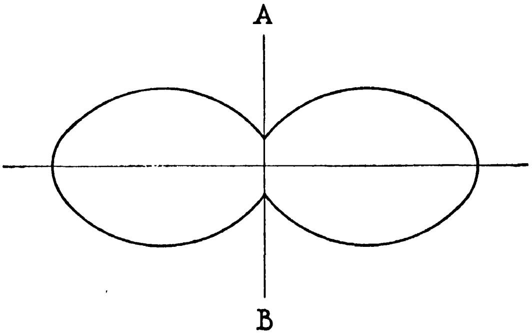

FIG. 3. The cross-section of the solid of revolution described in the text

$$x = \int_0^y f(y)\,dy$$

by graphical integration between suitable limits.

The best result which can be obtained is shown in Fig. 3, and for this curve the constants have the values: $\lambda = -0.5$, $\mu = 0.72$, $\theta = -0.8$. This curve, if rotated about its minor axis, gives the solid of revolution which is of the form required by the initial conditions. I scarcely need to point out that the form is suggestive of the shape of the mammalian erythrocyte.

But suggestive as the form may be, it represents an impossible state of affairs, for it has points on its surface, and at these points the tensions would be infinite. If one feels inclined to round off the points, one has to remind one's self that by doing so one brings the whole proof to the ground. It is for this reason that I refer to the result as disappointing and meaning very little. But the fundamental reasoning is sound: what has gone wrong is that we have had to put $y = 0$ when $x = x_0, x_1$ in order to obtain the differential equation. What we need to do is to put, not two points, but two finite curvatures on the surface, or, better, to put four points on the surface, one about the middle of each quadrant of arc, and then to replace them by finite curvatures. In fact, we have to deal with a real membrane with physical properties instead of with a mathematical surface. I am assured that this is beyond the powers of existing analysis. Nevertheless the reader has only to refer to Section I in order to see that there is a problem to be solved, and to re-read Section II in order to see that its contents represent an abortive attempt to solve it.

III

Supposing Norris' theory to be substantially correct, it is interesting to consider the kind of system to which it could apply. The theory demands only one condition, *viz.* that the surface of the cell shall exceed the minimum surface for the enclosed volume, and therefore there must be an "expansive force" operating somewhere. This force is often referred to as a "force of negative surface tension" without any clear idea as to how it is developed; we shall therefore consider it in some detail.

1. Consider first a drop of a homogeneous fluid in another homogeneous fluid. Surface tension forces act at the interface so as to bring the surface to a minimum for the enclosed volume, and the form of the drop is that of a sphere. The surface tension forces, however, are opposed by forces within the drop, which suffers a certain compression as a result of the operation of the surface forces; the latter are therefore opposed by an expansive force acting equally in all directions, and due to the fact that the molecules of the drop resist compression. The most familiar evidence of the existence of this expansive force is that the volume of the drop increases if the surface tension falls, and that the vapor pressure inside the drop exceeds that outside it by $2\sigma/r$.

2. Next take an imaginary case in which the molecules of the drop

repel each other in one direction more than in another. The result of such a state of affairs would be a greater pressure in one direction than in another, *e.g.* along an equator, and a flattening of the droplet with an increase of the surface above the minimum for the enclosed volume. Here again the forces of surface tension would be operating against an expansive force generated by a repulsion of the molecules of the drop. The difference between this case and that immediately above would be that the surface would be the minimum for the enclosed volume in case 1, and in excess of the minimum in case 2, but this would not be due to the latter system being subjected to a force essentially different from that acting in the former. The expansive force would be present in both, but in the latter it would act to a greater extent in one direction than in another, while in the former it would act equally in all directions.

The idea that the molecules in the cell interior repel each other to a greater extent in one direction than in another is precisely Gough's modification of Norris' theory. It is not impossible in itself, for quite a number of fluids exist in a mesomorphic state. The mesomorphic state, however, is usually most manifest at surfaces, and so we can go on to consider the next case.

3. Suppose that we have a cell membrane investing a homogeneous drop, the whole floating in a homogeneous fluid. The molecules of the membrane are constrained to remain in its plane, and, if they repel each other, the repulsive force will act in this plane. The force of surface tension, acting at the interfaces at the inside and at the outside of the membrane, will tend to produce the smallest surface for the enclosed volume, but will be opposed by the repulsive, or expansive, force just mentioned, the nature of which is essentially the same as it is in cases 1 and 2. Equilibrium will be reached when the surface forces are balanced by this expansive force, and at equilibrium the free energy will be at a minimum, although the surface may not be the minimum for the enclosed volume.[1]

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[1] This idea can be illustrated by a simple analogy. Consider a steel spring. Work has to be done to extend it, and also to shorten it, and in its uncompressed and unexpanded state the free energy is at a minimum. The fact that minimal free energy corresponds to a certain length of the spring is determined by the structure of the metal, and the theory which determines what this length must be is one which deals with molecular structure.

In each one of these cases the expansive force has the same origin, *viz.* molecular repulsion in the body of the drop or in its surface membrane, and the only difference between case 1 and cases 2 and 3 is that in the latter the force does not act equally in all directions. It is exceedingly unfortunate that this expansive force has come to be known as a force of negative surface tension, when the only thing which it has to do with surface tension is that it opposes the latter. The term "negative surface tension" has led to a confusion of ideas which, in turn, leads to most misleading results, as an extreme instance of which I may mention Volkonsky's revision of the physical theory of phagocytosis (see Volkonsky, 1933, and Mudd, 1933).

Norris' theory thus demands that there be a mutual repulsion between the molecules situated in the red cell membrane. It is not difficult to see how such a repulsion might arise, for hydrocarbons with a polar group directed towards the water (COOH groups, for instance, as in lecithin) might be mutually repulsive. Reference to Section I and to Fig. 2, will show, however, that the tensions along the membrane must vary from place to place (if the pressures are the same), and so we find that Norris' theory also demands that the (molecular) structure of the membrane shall not be homogeneous. This is tantamount to saying that the membrane has a "liquid crystal" structure. If so, the membrane cannot be made up of a homogeneous fluid.[2]

This hypothesis, *viz.* that the red cell membrane has a special molecular structure, is not one which can be rigorously tested at the present time. Some light may be thrown on it, however, by considering the conditions under which the special shape of the erythrocyte is changed.

(*a*) When the cell is acted on by lysins the molecular structure of the membrane is presumably broken down. Under such circumstances the cell always becomes spherical. (For a description of this and other changes in form, see Ponder, 1934.)

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[2] This idea, as opposed to the idea that the membrane is a region in which monomolecular films exist, is quite in keeping with the little which we know about the structure of cell membranes. Fauré-Fremiet, in particular, has shown that the "petaloid" pseudopodia of lymphocytes are composed of films not unlike those of soap films, in which the molecules are arranged in groups of varying thicknesses, and not in monolayers. A complicated "liquid crystal" structure of the cell membrane is therefore less unlikely than it appears at first sight.

(*b*) When the medium surrounding the cell is treated with lecithin, the cell becomes spherical without change in volume. The reason for the change in shape is obscure, but one cannot help associating it with the theory that the discoidal shape is due to a repulsion of lecithin molecules in the membrane.

(*c*) If the cell is in saline and is placed between a slide and a closely applied cover-glass, it becomes spherical without change in volume. Under such circumstances a pressure is developed, because the two glass surfaces are urged together with a force $= 2\pi r^2\sigma/d$ where r is the radius of the drop between the plates which are distance d apart, and where σ is the surface tension (fluid-air) of the liquid. Numerically the effect is about the same as that which would be produced by placing 150 gm. on the cover-glass. Again the effect is obscure but it is at least possible that such a pressure might affect a molecular configuration in the cell membrane.

(*d*) The form of the cell is also largely dependent on the presence of the plasma proteins in the fluid bathing it. If these are removed, crenation occurs, and if the proteins are present the cells will not become spherical between two glass surfaces. In this respect at least the red cell is similar to the myelin forms of lecithin, the shape of which is also largely dependent on the nature of the fluid surrounding them.

These observations, of course, prove nothing, but they show that the shape of the cell may be modified by factors such as might be expected, on *a priori* grounds, to modify a special molecular configuration within the membrane. At the same time it is interesting to observe that factors which might be expected to change the surface tension at the red cell surface have no particular effect on the shape. The shape is essentially the same, for instance, in isotonic citrate as in plasma, and is not materially altered when the cells are suspended in solutions of surface-active lysins (at least up to a short time before hemolysis), or in solutions of non-lytic surface-active substances, such as emulsin or the brominated saponins.

IV

The fact that we cannot find, from first principles, a formula for the surface of the red cell or for the curve which bounds its cross-section need not deter us from finding an empirical one. This can be done by plotting the cross-section between the limits of $+\pi$ and $-\pi$, and then applying Fourier analysis. The short method given by Whittaker and Robinson (1929, p. 271) is sufficiently accurate.

Owing to the symmetry of the curve, the coefficients of the sine terms are zero, and the series turns out to be

$$U = a_0 + a_1 \cos x + a_2 \cos 2x + a_3 \cos 3x$$

The following table gives the values for the coefficients for the cells of man, the rabbit, and the sheep, these being the only animals for which we have fairly reliable measurements.

	a_0	a_1	a_2	a_3
Man	33.7	−1.0	−16.2	−9.5
Rabbit	30.3	−4.0	−10.2	−8.5
Sheep	49.0	−8.0	−20.3	−9.5

The curve given by this series fits the cross-section of the cell almost to perfection. The empirical expression has, of course, neither theoretical significance nor immediate practical importance, nor does it offer any advantage in the calculation of either area or volume, but it may prove to be exceedingly useful when the time comes for a detailed investigation of changes in shape of the red cell under various conditions, especially as the standard errors of the Fourier coefficients are known if the standard errors affecting the data are known, as they would be in practice.

SUMMARY

This paper is concerned with an attempt to put Norris' theory for the shape of the mammalian erythrocyte into a quantitative form. The theory supposes that the biconcave form of the cell is brought about by an expansive force enlarging the surface, and is also supposed to apply to the formation of the myelin forms of lecithn. The attempt is not successful, and is published merely because it is suggestive. Various points regarding the shape of the cell, the curvature of its surface, and the kind of system to which Norris' theory might be supposed to apply, are discussed, and an empirical formula is given for the curve which bounds the cross-section of the cell. This empirical formula describes the shape almost to perfection.

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THE POTASSIUM EQUILIBRIUM IN MUSCLE

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Considering the enormous amount of work which has been done in an effort to unravel the physiology of muscle it is surprising that so little is known concerning its physicochemical organization, which is quite as fundamental to an understanding of its function as is its histological structure. According to our present imperfect understanding, as outlined particularly by Mond and Netter (1930, 1932), the potassium in muscle is largely inside the fibres where it is 30 to 40 times as concentrated as in the tissue spaces. All of this potassium inside the muscle must be in solution if the osmotic pressure inside is equal to that outside (Hill and Kupalov, 1930). When the muscle is injured or dies potassium escapes. The potassium inside, K_i, is in equilibrium with the potassium outside, K_o, so that when K_o is increased K_i also increases and when K_o decreases K_i likewise decreases. The equilibrium concentration of potassium outside is 13 mg. per cent. The muscle is believed to be permeable to cations as small as potassium but impermeable to sodium, which, because of its hydrated ion, is too large to pass the pores of the membrane. In consequence of its negative charges the membrane is impermeable to anions. The chloride of the muscle and an equivalent amount of sodium is believed to be in the tissue spaces because it is so readily removed by isotonic sugar, while the excess of sodium over chlorine is possibly combined in the surface of the fibres in some way. According to the theory of Mond and Netter the diffusion of potassium across the membrane in either direction as a result of a disturbance of the equilibrium will take place by exchange with hydrogen ions. Or, the potassium might diffuse in or out as undissociated KOH as suggested by Osterhout's theory (1930). On either theory the potassium change and the acid-base change should be equal. The following experiments were designed to test this relationship and to learn more concerning the changes in potassium concentration which can be produced experimentally.

The general scheme of the experiments as finally worked out was to soak thin sartorius muscles of the frog in isotonic solutions of varying compositions and to analyze matched muscles before and after the treatment for their contents of potassium and other ions. We preferred this simple technique, after trying the perfusion method of Mond and Netter, because it gave, in our hands, more uniform results and was much easier and quicker. Diffusion was of course slower, but the changes produced in 5 hours are easily measurable, and it is difficult to perfuse two frogs in a comparable manner, to be sure that every muscle in the frog is equally irrigated, and to know the exact weight of muscle tissue involved in the exchange of ions.

Method of Analysis

The muscles were placed in small covered platinum crucibles in an electric oven overnight and were thus reduced to a white ash. Known amounts of potassium submitted to the same procedure showed no loss. The oven temperature did not exceed 650°C. and attained this figure only after 3–4 hours. This is the method recommended by Ernst and Barasits (1929). The analysis of the ash was carried out by the platinic chloride precipitation as described by Shohl and Bennett (1928).

Two drops of N/1 hydrochloric acid and 0.3 cc. platinum chloride (10 per cent) were added to the ash in the crucible and mixed thoroughly, 5 cc. absolute alcohol were added, and the mixture was allowed to stand for 20 minutes. Then the crucible was transferred to a Jena glass filter funnel in a suction flask and inverted. Precipitate, filter, and crucible were washed on the filter four or five times with alcohol saturated with K_2PtCl_6, and then three or four times with 10 per cent KCl, also saturated with K_2PtCl_6. The funnel was transferred to a clean suction flask, and the funnel and crucible washed with 10 or 15 cc. of hot water; then with 1 cc. of 2N KI and more hot water, the final volume being 25 or 30 cc. The flask was incubated at 65°C. for 10 or 15 minutes and titrated hot with $Na_2S_2O_3$ (about 0.005 M) from a micro burette calibrated to 0.02 cc. The technique was checked by an analysis of a known solution each day. The analyses were not continued until an accuracy of 2–3 per cent was attained.

Muscles were weighed on a torsion balance before and after immersion. Unless otherwise stated the weight changes during immersion were not important and concentrations were calculated per gram of initial wet weight. Irritability was measured in most cases after immersion in the experimental solution. This was done by determining the minimum primary current in an induction coil which would just cause a contraction. The coil distance was kept at zero and the results are expressed as reciprocals of the primary current or ohms/volt. The current was regulated by a variable resistance. Only at very low resistances did this method lead to difficulties on account of the variable electric resistance of the cell itself.

The pH of the solutions was checked by use of a quinhydrone electrode using 0.05 M potassium phthalate as standard.

The muscles were handled by a thread tied to the tendon. They were exposed to 100 cc. of the solutions in 250 cc. Erlenmeyer flasks in a water bath at 22°C. The flasks were fitted with two-hole rubber stoppers and two tubes, one reaching to the bottom for use in equilibrating the mixture with 5 per cent CO_2 or other gas mixture. The CO_2 was measured into a spirometer from a burette and the final mixture checked by gas analysis. Unless stated to the contrary all the solutions contained M/150 phosphate buffers, $CaCl_2$ 0.02 per cent, and NaCl 0.65 per cent. The NaCl content was decreased to compensate for large amounts of KCl or $NaHCO_3$ added to attain the desired pH in the presence of CO_2.

RESULTS

Normal Variations in Potassium Content.—Over a thousand sartorius muscles have been analyzed for potassium during this investigation.[1] Some of these analyses were done in matched untreated muscles merely to test the accuracy of the technique. In one series, eleven pairs of semitendinosus muscles were analyzed. The results for two of these pairs differed by 15 and 23 per cent respectively but in the remainder the maximum difference between any two matched muscles was 6.2 per cent and the average difference was 3.2 per cent. In a second series of fifteen muscles one pair differed by 24 per cent but the maximum difference in the other fourteen pairs was 8 per cent while the average difference was 2 per cent. An occasional analysis is therefore erroneous, or an occasional pair of muscles widely divergent.

The potassium content of 89 sartorius muscles was 330 ±23 (probable error) mg. per cent (mg. K per 100 gm. wet weight of muscle), while 45 other leg muscles, mostly thigh muscles, gave an average value of 316 ±23 mg. per cent. The average of all 134 analyses was 325 mg. per cent.

No significant seasonal variations could be established between the months of October and May nor any constant differences between sartorius, gastrocnemius, etc. Nevertheless we believe that there are important differences between different muscles in the same frog even though the differences are not in the same direction in all frogs. Thus in five frogs, both sartorius, both semitendinosus, and both semimembranosus muscles were analyzed. The *average* deviation from the sartorius value in the same frog was 24 per cent, while the average difference between the two muscles of each pair was only 2 per cent, as stated above. It is not therefore justifiable to analyze a gastrocnemius muscle as a control for a sartorius muscle. These results indicate that a pair of sartorius muscles must be used for each experiment, one for each of two solutions to be compared or else one for the initial potassium content and the other for the change produced by the experimental procedure.

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[1] All the analyses were made by the junior author. We are indebted to Miss Margaret Erlanger for some preliminary analyses.

Usual care was exercised in dissecting the muscles to avoid injury and the dissection was carried as close as possible to the origin. Nevertheless there seemed a possibility that a large fraction of the potassium interchange between muscle and solution might take place at the ends of fibres cut across at the pelvic end. To test this possibility six pairs of sartorius muscles were cut in halves in the middle, the two pelvic and the two tibial ends from each pair being analyzed together. Six other pairs were similarly treated after soaking for 5 hours in Ringer's solution. The results are shown in Table I.

TABLE I

	K	
	Pelvic half	Tibial half
	mg. per cent	*mg. per cent*
Controls	317	334
After 5 hrs. in Ringer's	324	321

The differences are probably not significant but such differences as do appear would suggest that the loss of potassium was more rapid through the slightly smaller tibial half than through the slightly injured pelvic half. Hence it may be concluded that the loss through the injured end is not excessive.

Varying Potassium Concentration.—

A series of isotonic solutions was prepared in 250 cc. Erlenmeyer flasks with concentrations of potassium varying from zero up to 196 mg. per cent. In addition to the potassium, each solution contained $CaCl_2$ 0.02 per cent, NaH_2PO_4 M/150, and NaCl in amounts which decreased as KCl increased. Each solution was equilibrated with oxygen and contained enough NaOH to give the desired pH, in this case 5.6 and 7.2. One sartorius muscle was placed in each solution, the matched muscle from the same frog being analyzed at once for potassium. The flasks were left in the cold room at about 4°C. for 22–23 hours when the muscles were removed, superficially dried on filter paper, weighed on a torsion balance and put into a crucible for ashing and potassium analysis. In this experiment, the potassium content was calculated on the basis of the final weight of the muscle after immersion.

Fig. 1 shows the results obtained in two experiments of this type, one at pH 7.2 and the other at pH 5.6. Ordinates represent the differences in potassium content between the control and experimental muscles. At pH 7.2, the graph crosses the zero line at 19 mg. per cent indicating that in a solution of this concentration and under the conditions of this experiment a muscle will neither gain nor lose potassium.

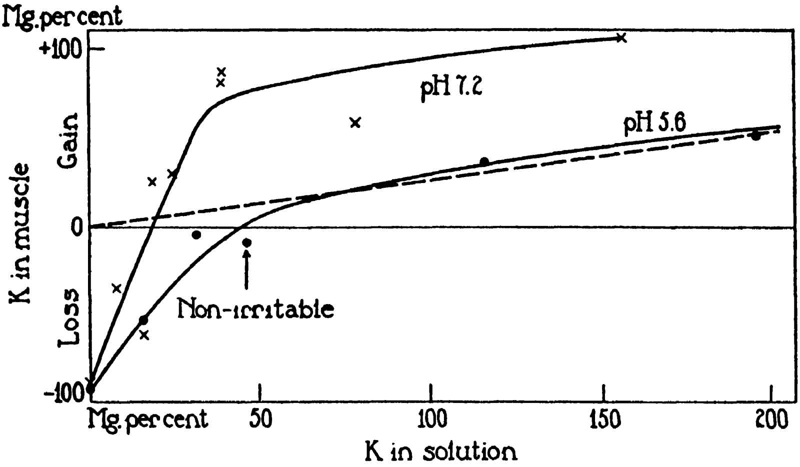

FIG. 1. Graphs showing the gain or loss of potassium by a frog sartorius muscle after immersion for 22–23 hours at 4°C. Each point represents the difference in potassium content between the control muscle analyzed before the period of immersion and the experimental muscle analyzed after immersion.

In weaker solutions there is a loss of potassium from the muscle and in stronger solutions there is a gain.[2]

At pH 5.6 a more concentrated solution is required to prevent the loss of potassium, the equilibrium concentration being 46 mg. per cent. At concentrations larger than this there is as before a gain in potassium but in this experiment the gain is no larger than would be expected if only the tissue spaces gained potassium until the concentration equalled that in the solution outside, the potassium content of the fibres themselves remaining unchanged. Assuming 25 per cent of the whole muscle as tissue space and neglecting the small amount of potassium originally present therein, the gain in potassium to be expected from this cause has been calculated and is shown as a dotted line in Fig. 1. Thus at 200 mg. per cent K_o the increase in K_i would be 50 mg. per cent. The observed gain is evidently well explained by the tissue spaces and there is no reason to suppose therefore that the potassium concentration inside the fibres has increased.

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[2] Jacques and Osterhout (1932) have reported similar findings in *Valonia*.

In these very strong potassium solutions the muscle also swells, due presumably to a loss of the normal anion impermeability. At pH 5.6 the gain in weight was 12 per cent and 13.3 per cent at 117 and 196 mg. per cent K respectively. If the potassium contents had been calculated in per cent of the initial weight of the muscle the gain in potassium in these solutions would have been much larger than that indicated but the actual concentration of potassium in the fibres might have been decreased. Since we are interested in concentration rather than contents we have calculated potassium according to the weights after immersion in all solutions which cause a swelling of the muscle.

As long as the potassium inside the muscle is in equilibrium with the potassium outside, an increase in K_o should lead to an increase in K_i. According to Fig. 1 this occurs up to a certain point after which there is no further increase in K_i. This point of inflection in both curves seems to coincide fairly accurately with the point indicated by an arrow, at which the muscles, at both pH 5.6 and 7.2 become almost completely non-irritable. While more experiments are necessary to establish this correlation as strictly valid, it appears that when the muscle membrane is injured by the high potassium concentration to such a degree that excitation becomes impossible, the concentration of potassium inside the fibres has reached a limit. Possibly the membrane has become permeable to anions. This would result in swelling also if the protein anions are still unable to escape. It is in fact only in the solutions stronger than 100 mg. per cent K at both pH 7.2 and 5.6 that swelling was observed. At pH 7.2 there is, however, a large increase in the concentration of potassium before this point is reached.

Brief mention may be made at this point of the effect of immersing muscles in isotonic KCl (0.9 per cent or 472 mg. per cent K). Such muscles gain in weight at the rate of 16 per cent of the initial weight per hour for about 16 hours and then remain about constant. The solution which is absorbed is approximately isotonic as regards potassium but contains much less chloride. Since the muscle as a whole becomes simultaneously slightly more acid and since the loss in sodium does not cover the excess of potassium over chlorine, it is obvious that some other ions than K, Na, H, OH, and Cl must be involved. Lactic acid formation and phosphocreatine synthesis help to account for the remainder of the potassium. Some of the potassium absorbed serves merely to raise the concentration in the tissue spaces. Some of our analyses show more potassium absorbed than can be accounted for in this way, indicating an increase in concentration inside the fibres, but this seems to be the exception rather than the rule. With the irreversible loss of excitability of the muscle has gone also, to a large extent at least, the ability to concentrate potassium.[3]

The Effect of pH

Perhaps the most significant feature of Fig. 1 is the fact that at pH 7.2 the tendency of potassium to escape from the muscle is less than at pH 5.6; a smaller value of K_o is necessary to prevent decrease in K_i. This is to be expected on the basis of a membrane equilibrium if the membrane is permeable to potassium and indeed Höber (1928) has some observations to confirm this prediction. Likewise Ernst and Takacs (1931) show that the addition of lactic acid to the sugar perfusate increases the amount of K and PO_4 which is extracted from frog muscle.[4] It seemed possible, however, to test the matter more quantitatively by determining the effect of pH upon the equilibrium potassium concentration; *i.e.*, the concentration of K_o at which the muscle neither loses nor gains potassium. Thus if the equilibrium is such that $K_i/K_o = H_i/H_o = OH_o/OH_i$, where K_i and K_o refer to the activities inside and outside the muscle respectively, then $K_i \times OH_i = K_o \times OH_o$.

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[3] In a preliminary abstract of this work we did not allow for the swelling of the muscle and concluded, probably erroneously, that a muscle could continue to concentrate potassium even after irritability is lost (Fenn, 1933).

[4] Recent experiments performed since writing this manuscript have indicated that the loss of potassium is to a considerable extent dependent upon the ability of the muscle to lose phosphate. Matched muscles were soaked in high (M/150) and low (M/3000) phosphate Ringer without potassium. In the latter solution phosphate would diffuse out (Stella, G., *J. Physiol.*, 1928, **66**, 19) and the loss of potassium from the muscles in two experiments was 73 and 90 mg. per cent greater than in the more concentrated solution. We have also found a 19 mg. per cent greater loss of K from a muscle in nitrogen than from its mate in oxygen possibly because of the simultaneous loss of lactate. We were unable to confirm the statement of Wojtczak (Wojtczak, A., *Biol. Abst.*, 1929, **3**, 19068) that absence of sugar from the Ringer's solution accelerates the loss of potassium.

If at equilibrium, *i.e.* when K_i is constant, OH_i is also constant, then $K_o \times OH_o$ should be constant. In other words as the pH is changed, the equilibrium value of K_o should change in such a way that K_o and OH_o should remain constant and equal to $K_i \times OH_i$.

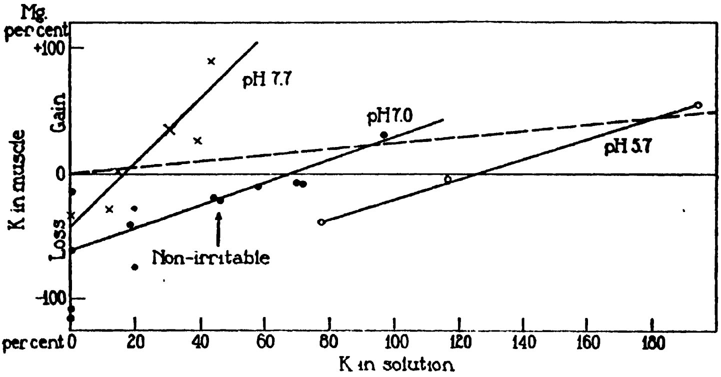

FIG. 2. Gain or loss of potassium at different pH after immersion for 5 hours at 22°C. in 5 per cent CO_2-bicarbonate-phosphate buffers.

To test this suggestion it was necessary to determine the "equilibrium points" in a number of solutions of different pH values. A pH of 6.3 was tried under conditions comparable to those shown in Fig. 1 and also a series of similar experiments under different conditions in the hope of obtaining more uniformity in the behavior of different muscles. For this reason some muscles were left in the cold room overnight in aerated Ringer's solution to reach a "basal" state and to recover from dissection injuries. The most carefully studied condition, however, was at 22°C. in an atmosphere of 5 per cent CO_2 + 95 per cent O_2 with M/150 phosphate and bicarbonate to give the desired pH. This offered the further advantage that the bicarbonate content of the muscle could be measured simultaneously, thus permitting a calculation of the pH inside the muscle. The data obtained from some of these experiments are plotted in Fig. 2. Other series at pH 6.3, 7.25, and 7.5 were tried and have given intermediate and confirmatory values which will be omitted to avoid undue confusion. The results are essentially similar to those of Fig. 1 except that the equilibrium concentrations are uniformly larger, due chiefly to the higher temperature, and no definite point of inflection is observed corresponding to the approximate point of non-irritability, although this might have appeared in still higher concentrations at pH 7.7.

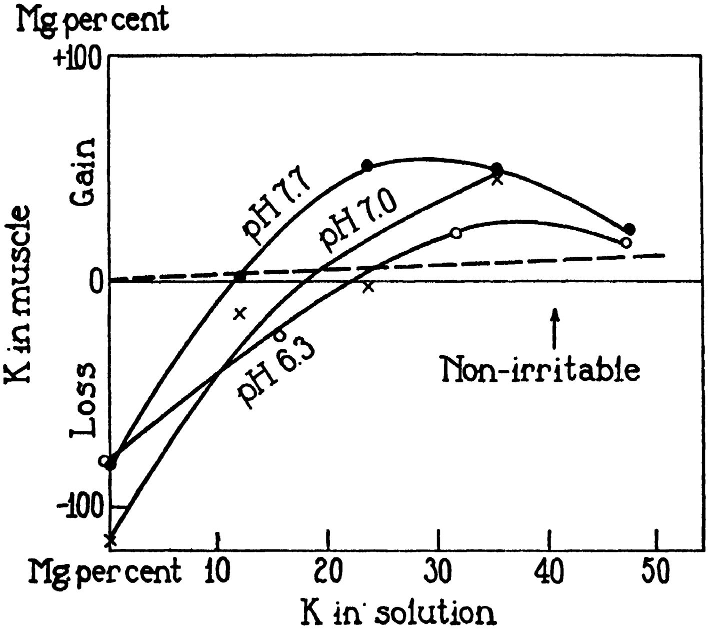

FIG. 3. Same as Fig. 2 but the muscles were immersed for 22 hours at 4°C.

Three more curves of the same type are plotted in Fig. 3. The muscles were immersed in the same solutions described in connection with Fig. 2 but they were kept for 22 hours in the ice box at about 4°C. in the hope of diminishing the rate of metabolic disintegrative changes and leaving the diffusion rates relatively unchanged. There is much less difference between pH 6.3 and 7.7 than in Fig. 2. In the alkaline solution there is a definite increase in the concentration of potassium at least up to a certain limit. Beyond this maximum at pH 7.7 there is a decrease in potassium concentration which may be real and may be correlated as before with the diminishing irritability found in these solutions. It should be noted that in this series even at pH 6.3 there is definite evidence of a movement of potassium into the fibres against the concentration gradient. According to this test the anion impermeability still persists and to that extent the cell is still in a normal condition.

The irregularity of these results is considerable[5] but the conclusions are unmistakable. It is obvious that it does not suffice to say that the muscle is in equilibrium with a solution containing 13 mg. per cent potassium, for this varies markedly with the pH. Mond and Amson (1928) determined this value by perfusing with a solution containing $NaHCO_3$ but no PO_4 or CO_2 except such CO_2 as came from the metabolic processes in the muscle.[6] The arterial solution must therefore have been quite alkaline which would agree with our results.

Since K_o (at equilibrium) decreases with increase in pH according to theory we have next to inquire whether this decrease is quantitatively as well as qualitatively in agreement with the theory.

This can best be tested by plotting log K_o against log OH_o (or pH). If $K_o \times OH_o$ is equal to a constant then log K_o + log OH_o or log K_o + pH is equal to a constant and the graph should be a straight line of such a slope that log K_o should decrease from (say) 2.0 to 1.0 when pH increases from 6.0 to 7.0. The slope of the graph should be therefore independent of the absolute value of the product $K_o \times OH_o$ which will determine only the intercepts on the x and y axes.

In Fig. 4 the "equilibrium" values of log K_o and log OH_o (pH) (*i.e.* when K_i is constant) have been plotted in this way using all the experiments available of the type described in Figs. 1–3. Five graphs, A, B, C, D, and E are obtained. The theoretical slope for constancy of the product $K_o \times OH_o$ is indicated by straight lines. It is evident that the most thoroughly studied condition (5 per cent CO_2, 5 hours, at 22°C.) represented by Curve A, gives results which approximate fairly well to this slope within the physiological range from 7.0 to 7.7. Parts of Graphs B, E, and D also seem to approach this slope as a limiting value. At pH below 7.0, Graph A deviates markedly from the theoretical slope. This we believe due to the fact that the pH does not remain constant inside the muscle even though K_i is constant. If the product $K_i \times OH_i$ varies there is therefore no reason to expect $K_o \times OH_o$ to remain constant. Instead of remaining constant at pH

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[5] We have made no attempt to select typical experiments for presentation but have included practically all the results obtained. Many more experiments would be needed to determine the extent of the variations which could be expected in the behavior of frog muscle.

[6] These authors mention no pH effects in their perfusion experiments but in a personal communication Dr. Mond informs us that they looked for such effects but did not observe them.

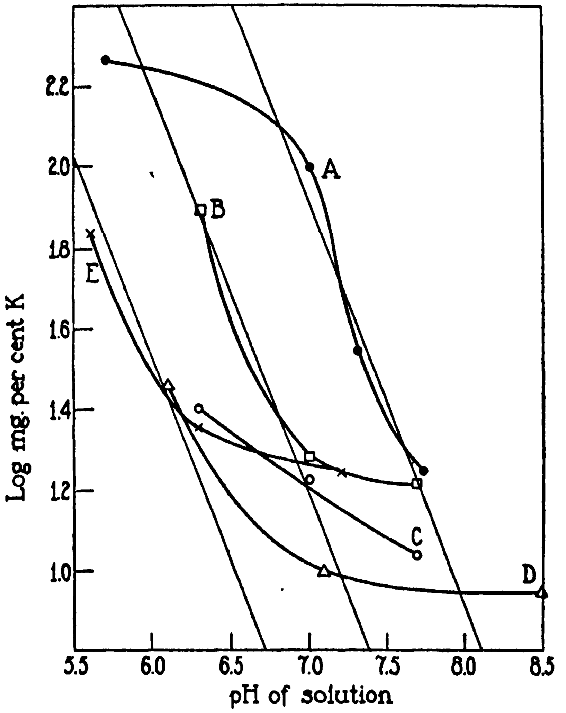

FIG. 4. The pH of the solution plotted against the logarithms of the corresponding potassium concentrations at which the muscles neither gain nor lose K. See text. The curves differ as follows: A, 5 per cent CO_2, 5 hours, 22°C. (Fig. 2); B, same as A but muscles previously kept overnight in Ringer's solution at about 4°C.; C, 5 per cent CO_2, 22 hours at 4°C. (Fig. 3); D, phosphate buffers, no Ca, 5 hours at 22°C. after previous treatment overnight at 4°C. in Ringer's solution; E, phosphate buffers, 22 hours at 4°C. (Fig. 1).

5.6 outside the pH inside decreases. This large concentration of H^+ inside readily exchanges with K outside. A smaller concentration of K_o is therefore necessary to keep K_i constant.

We do not offer Fig. 4 as proof that $K_o \times OH_o$ is constant. The points are hardly accurate enough to prove that this and no other relation obtains. Furthermore the pH inside is not really constant anywhere in the physiological range except at pH 7.0. Fig. 4 does show, however, that the data are not inconsistent with this theoretical interpretation. One of the chief difficulties with the theory is that the pH inside is not much if any less than pH outside. To demonstrate this fact it is necessary to describe measurements of the pH inside the muscle.

pH Changes inside the Muscle.—The pH inside the muscle was estimated from the combined CO_2 when equilibrated with a known CO_2 tension (5 per cent) by means of the Henderson-Hasselbalch equation. On account of the doubtful assumptions involved in using this equation for tissues, especially the impossibility of making allowances for tissue spaces, the resulting figure is properly referred to as an "equivalent pH."

The H_2CO_3 in 5 per cent CO_2 per 100 gm. of muscle was taken to be $0.829 \times 0.80 \times 0.05 \times 100 = 3.316$ vol. per cent assuming 80 per cent water in the muscle, and taking 0.829 as the absorption coefficient for CO_2 in water at 22°C. For 5 per cent CO_2 the pH is therefore calculated by the formula $\text{pH} = 5.65 + \log$ (vol. per cent combined CO_2) when $pK_1 = 6.17$. The combined CO_2 is also calculated per 100 gm. of muscle. If both HCO_3 and H_2CO_3 were calculated per 100 gm. of muscle water the result would of course be unchanged. The value of pK_1 was taken from Cullen, Keeler, and Robinson (1925) for human blood plasma at 22°C. According to Meyerhof, Möhle, and Schulz (1932) the pK_1 should be greater than that for bicarbonate by 0.14 on account of a difference in the activity of HCO_3^- in muscle. These authors use $pK_1 = 6.40$ at 20°C. If this value were used it would be necessary to conclude from our experiments that a muscle which neither lost nor gained CO_2 in 5 hours in a solution of pH 7.0, had a pH inside of 7.23. The pH of our solutions was measured with a quinhydrone electrode using a potassium phthalate standard.

The combined CO_2 was measured as follows: The muscle was removed from the experimental solution, blotted on filter paper, weighed, and placed in 1 cc. of unbuffered Ringer's solution in a respirometer bottle (differential volumeter) containing 0.3 cc. of 25 per cent citric acid in a side arm. 5 per cent CO_2 + 95 per cent O_2 was passed through the bottle, the solutions having been previously equilibrated with the same gas. Cocks were turned after the respirometer had been in the bath 10 minutes, and half an hour later the acid was dumped into the Ringer's solution. Readings were continued after acidification until there was no further increase in volume. Ordinarily the volume begins to decrease again very slowly within 1 hour after dumping. A correction was of course made for the solubility of CO_2 in the Ringer's solution and in the muscle on the basis of the absorption coefficient of CO_2 for water at 22°C.

The results of these measurements show us what changes to expect in the position of the CO_2 dissociation curve previously determined for normal muscles (Fenn, 1928). They have a further special importance in furnishing a test of the theory that transfer of potassium takes place only by exchange with hydrogen (or by diffusion as KOH). In either case, since the muscles are equilibrated with 5 per cent CO_2 during immersion in the experimental solutions, any penetration of potassium by either of these mechanisms would cause an equivalent increase in combined CO_2. The results show that this takes place to a limited extent only.

Muscles were soaked for 5 hours in solutions of varying pH with Ca, K, PO_4, and Na constant, CO_2 buffers being used. The results are shown in Fig. 5, all the muscles used in this series being represented. The "equivalent pH" inside is plotted against the pH outside, the diagonal through the origin representing the points where the pH is the same inside and outside. The normal muscle has an initial pH in 5 per cent CO_2 of 7.0 as indicated by the horizontal line. As diffusion takes place in the experimental solution the pH inside decreases in acid solutions and increases in alkaline solutions as if the initial horizontal line were gradually rotating anticlockwise, as indicated by the arrows, to approach the diagonal line. After 5 hours the combined CO_2 is 8–10 vol. per cent at pH 6.3 outside and 40–50 vol. per cent at pH 7.7, the normal content in HCO_3 being about 22 vol. per cent. These changes are large and relatively independent of small effects due to differences in potassium content of the solution. This is evident when it is realized that the potassium concentration in some of the solutions varied at the same pH from 0 to 51 mg. per cent K. The pH inside, in other words, is almost entirely dependent upon the pH outside and the period of immersion. Evidently *the pH inside can change independently of the potassium which is therefore not the only penetrating ion.*

It is remarkable that an inside pH of 7.0 should be exactly in equilibrium with a pH outside of 7.0. The same is true of nerve except that the pH concerned is 7.15. With potassium 30 times more concentrated inside than outside there should be, in a Donnan equilibrium a like difference in H^+, so that the pH inside the fibres themselves ought to be 5.62 if it is 7.0 outside. The theory is evidently incomplete.

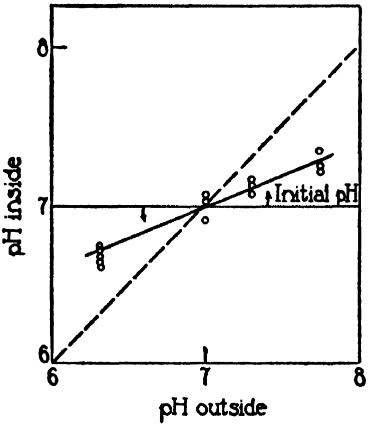

FIG. 5*a*

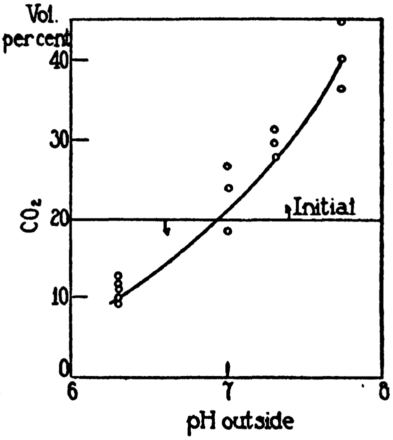

FIG. 5*b*

FIG. 5*a*. Calculated value of pH inside the muscle after 5 hours immersion at 22°C. in solutions of different pH using 5 per cent CO_2-bicarbonate-phosphate buffers.

FIG. 5*b*. The combined CO_2 contents from which the pH values of Fig. 5*a* were calculated.

Effect of Varying Potassium Concentration in the Solution upon the pH inside

If potassium enters a muscle by exchange with a hydrogen ion or as KOH, then a high potassium content in the solution should cause an increase in pH inside. In other words the pH_i should depend upon both pH_o and K_o just as K_i depends upon both pH_o and K_o. Experiments show that this is the case to a limited extent, although all the potassium which diffuses cannot be accounted for in this manner.

Matched muscles were soaked for 5 hours at pH 7.0, one in a solution containing no potassium and one in a solution of high potassium (43 mg. per cent) content. 5 per cent CO_2-bicarbonate buffers were used.

After 5 hours the muscles were blotted, weighed, put in a respirometer in 1 cc. of unbuffered Ringer's, and equilibrated again with 5 per cent CO_2 + 95 per cent O_2. The combined CO_2 was then determined by dumping citric acid and the pH calculated. The contents of the respirometers were finally rinsed into a crucible, evaporated to dryness, ashed, and analyzed for potassium. The potassium originally present in the Ringer's solution was deducted from the total found.

TABLE II

Matched Muscles A and B in Ringer's Solution of High and Low Potassium Content at pH 7.0

	Experiment No.	Muscle A	Muscle B	Difference
				m.-eq. per 100 gm.
K in solution, *mg. per cent*		0	43	
CO_2 in muscle, *vol. per cent*	130	19.6	21.2	0.070
	132	18.3	21.1	0.126
	143	7.7	15.5	0.347
	144	8.8	12.8	0.179
Average				0.181
K in muscle, *mg. per cent*	130	240	332	2.35
	132	276	254	1.99
	143	320	402	2.10
	144	334	409	1.92
Average				2.09

There was no significant change in weight of the muscles in either solution during immersion. Temperature = 22°C.

The results of four such experiments are shown in Table II. Averaging the results together it is found that Muscle B after 5 hours in high potassium Ringer's solution contains 2.09 m.-eq. per 100 gm. muscle more K than Muscle A, and only 0.18 m.-eq. per 100 gm. more HCO_3. The calculated increase in pH inside due to the high potassium is 0.14 as an average. Some potassium can obviously have diffused out of Muscle A as KOH thus decreasing the $KHCO_3$ inside. Some potassium may have diffused out without a corresponding decrease in HCO_3^- if accounted for by the buffer activity of phosphates, proteins, etc. The fraction of the excess of potassium in Muscle B which could be explained in this way, however, is very small as can be estimated from the CO_2 dissociation curve of frog muscle (Fenn, 1928). From this curve (for summer frogs) it can be ascertained that between pH 6.8 and 7.2 the combined CO_2 increases 3.9 vol. per cent or 0.174 m.-eq. per 100 gm. muscle for 0.4 increase in pH. The average pH in Muscle B is only 0.14 greater than that in Muscle A. Hence the amount of KOH which could be buffered by this means is only $0.14/0.40 \times 0.174$, or 0.006 m.-eq. If therefore the difference between Muscles A and B is interpreted as due to diffusion of potassium from Muscle A it must be concluded that only $0.181 + 0.006$ or 0.187, out of the 2.09 m.-eq. which are lost, can have diffused out as KOH, unless some other acid-base change has occurred such as a formation of lactic acid or of phosphocreatine.

It will be observed in Table II that in Experiments 143 and 144 the CO_2 values are lower and the potassium values are higher than in Experiments 130 and 132. This difference is due to the fact that the former muscles were left in aerated phosphate Ringer for 15 hours at 4°C. before being used. During this time the CO_2 tension was near zero and the reaction must have been fairly alkaline inside. Presumably therefore base diffused out (or acid in). Later when the muscles were put into 5 per cent CO_2-bicarbonate solution at 22°C. the CO_2 diffused in and they became therefore more acid than normal muscles and had a low CO_2 content. Being more acid inside they lost less potassium during the treatment at 22°C. with no potassium in the Ringer's. The average pH of Muscles A and B respectively was 6.93 and 6.98 for untreated muscles and 6.57 and 6.80 for muscles left in the cold room. The untreated muscles, being in a solution of pH 7.0, did not change much from this value as already explained in Fig. 5.

The average weight of the muscles used in the experiments of Table II was 127 mg. (105–145). In the absence of potassium the muscles lost on the average 2.2 mg. in weight while in the high potassium solutions they gained 2.8 mg. The difference in weight is therefore about 4 per cent while the difference in potassium between the two muscles is 25 per cent. There must have been some compensatory changes in other ions, perhaps sodium. The weight was, however, consistently higher where the potassium content was high.

All the muscles in these experiments (Table II) were irritable at the end of the experiment but those in the high potassium solutions had an irritability of only 45 as compared to 278 (ohms per volt) for the low-potassium muscles.

The above interpretation of the effect of soaking muscles in the cold room overnight is rather instructive and is confirmed by an experiment in which one of a pair of sartorius muscles (A) was analyzed immediately after dissection for combined CO_2 and K, while its mate (B) was similarly treated after being left in the cold room overnight in normal aerated Ringer's solution. The potassium decreased from 356 to 288 mg. per cent while the HCO_3 decreased from 26.2 to 12.2 vol. per cent, the "equivalent pH" values for the inside of the muscle (after equilibrating with 5 per cent CO_2) being 7.07 and 6.74 respectively. The decrease in potassium in this case was 1.74 m.-eq. of which loss only 0.62 m.-eq. was accounted for as HCO_3.

TABLE III

Phosphate vs. Bicarbonate Buffers

Experiment	Solution		K in muscle	
	pH	K	PO_4	CO_2
		mg. per cent	*mg. per cent*	*mg. per cent*
A	6.3	0	178	224
B	7.3	47	257	370

Another experiment (A) summarized in Table III illustrates nicely the effect of the pH inside the muscle upon the loss of potassium. Two solutions were prepared containing usual amounts of NaCl, $CaCl_2$, and phosphate buffers, but no KCl. One was equilibrated with O_2 and the other with 5 per cent CO_2 + 95 per cent O_2. The pH of both was adjusted to 6.3. A pair of sartorius muscles was immersed, one muscle in each solution, and left in the cold room overnight. In this solution both muscles lose potassium, but the one in the solution containing only phosphate buffers lost 46 mg. per cent more than the one in the solution containing phosphate plus CO_2 buffers. The difference is to be attributed to the greater acidity inside the latter due to the 5 per cent CO_2. A similar experiment (B) was tried at pH 7.3 with 47 mg. per cent K. The initial potassium content is unknown but probably the CO_2 muscle gained potassium and the PO_4 muscle lost potassium, the difference in potassium content being 113 mg. per cent. These experiments show that an increase in pH inside favors the loss of potassium from the muscle just as an increase of pH in the solution favors the entrance of potassium into the muscle from the solution. In spite of this indubitable relation between pH and potassium diffusion, evidence has been cited in Table II to show that potassium is not the only diffusible ion, for pH changes can occur independently of potassium diffusion.

TABLE IV

Mutual Independence of K and HCO_3 Changes

pH	K in solution	K in muscle	HCO_3 in muscle
	mg. per cent	*m. mols/100 gm.*	*m. mols/100 gm.*
6.3	0	—	−0.69
6.3	23	−3.72	−0.95
6.3	51	−0.33	−0.62
7.3	0	−2.07	0.32
7.3	23	−0.13	0.094
7.3	51	1.43	0.23

The figures represent differences between K and HCO_3 analyses of pairs of muscles, one analyzed before and the other after soaking for 5 hours at 22°C. in 5 per cent CO_2 + 95 per cent O_2. Solutions contained 0.65 per cent NaCl, M/150 phosphate, 0.02 per cent $CaCl_2$ in addition to KCl and HCO_3.

Further evidence of the same effect is furnished by the data of Table IV in which figures are given for simultaneous changes in K and HCO_3 concentration caused by an immersion of 5 hours in solutions of varying potassium and hydrogen ion concentrations. At pH 6.3 muscles lose 0.62–0.95 m.-eq. HCO_3 per 100 gm. in solutions of all concentrations of potassium from 0–51 mg. per cent, but at 51 mg. per cent K the loss of potassium is only 0.33 while in the absence of potassium it is certainly more than 3.72 m.-eq. although the actual analysis in this case was lost. Likewise at pH 7.3 all three muscles gain HCO_3 while two of them lose K. Under other conditions they may lose HCO_3 when K is gained; they may show a large change in pH after immersion while K remains constant, or the K content may change while the pH remains constant.

Effect of NH_4Cl

Another instructive example of an increased loss of potassium due to a high internal pH is derived from experiments in which some of the NaCl in Ringer's was replaced by NH_4Cl. As in the case of the erythrocyte, NH_4OH readily penetrates the muscle and increases the alkalinity inside. This increased alkalinity causes in turn a redistribution of potassium between the inside and the outside so that potassium diffuses out, perhaps due to the increase in the product $K_i \times OH_i$.

TABLE V

Effect of NH_4Cl on K Content of Muscle

	K in muscle		Irritability	
	(*a*)	(*b*)	(*a*)	(*b*)
	mg. per cent	*mg. per cent*		
Control muscle in Ringer's................	367	316	600	370
Muscle in Ringer's + NH_4Cl..............	278	257	20	10

The amount of NaCl replaced by NH_4Cl was 0.033 M in (*a*) and 0.044 M in (*b*). In both cases the solution had a pH of 7.3 and a potassium content of 32 mg. per cent. Muscles were soaked in these solutions for 5 hours at 22°C. before analysis.

On this basis Jacques and Osterhout (1930) explain the loss of potassium from cells of *Valonia* exposed to NH_4Cl. It was also noted that the irritability of the muscles to break induction shocks decreased markedly as potassium diffused out. The figures from these experiments are shown in Table V.

Effect of $CaCl_2$

We have endeavored repeatedly to learn what effect a change in the calcium concentration would have upon the potassium equilibrium. The results, however, have not been altogether consistent. Nine such experiments may be selected for summary. Each experiment involved the analysis of a pair of muscles after 5 hours immersion, one in a Ringer's solution containing no calcium and the other in a Ringer's solution containing 0.02 or 0.04 per cent $CaCl_2$. Otherwise the solutions were the same for both muscles. The pH of both solutions varied, however, in different experiments from 4.5 to 7.5 and the potassium from 8 to 32 mg. per cent. Of the nine experiments, seven showed more potassium in the muscle in the $CaCl_2$-Ringer solution, the average increase being 53 mg. per cent. The other two experiments showed decreased amounts of potassium in the presence of $CaCl_2$, the differences being 19 and 50 mg. per cent. It appears therefore in general that $CaCl_2$ tends to prevent the loss of potassium from muscles but the differences are not great and there are doubtless complicating factors which could not be adequately controlled. We find it impossible therefore to make a very positive statement concerning the effect of calcium at the present time.

Correlations with Irritability

In most of these experiments we have measured the irritability of the muscles after and sometimes also before the period of immersion in the experimental solution. Some correlations with the potassium equilibrium can be made out but in other respects the correlation is not very good.

Immediately after dissection the irritability of a frog sartorius muscle measured with an induction coil is perhaps 1000 ohms/volt. If left in Ringer's solution this value rapidly declines within the 1st hour to about 100 or 200 after which the decline is relatively slow. Samples of the rapid decrease are shown in the two lower graphs in Fig. 6. One of these graphs represents normal muscles and the other represents muscles equilibrated with 30 per cent CO_2 and therefore much more acid both inside and out. Each graph represents the average of three or more experiments. To avoid confusion the large number of scattering experimental points from many different muscles are not included. As would be expected the greater acidity causes a more rapid loss of irritability. Some of the muscles were analyzed for potassium at various times during these experiments and the results, represented by the graphs in Fig. 6, show that the more rapid loss of irritability was accompanied by a more rapid loss of potassium. The more rapid loss of potassium is probably due to the fact that CO_2 causes initially a greater increase in acidity outside in the Ringer's solution than inside the muscle, because muscle is better buffered than is the Ringer-phosphate solution. The buffer value (*dB*/*d*pH) for muscle is 0.02 and for Ringer's solution 0.0058 (with M/150 phosphate). It may be estimated that in 30 per cent CO_2 the pH inside the muscle was 6.3 as compared to 5.9 outside. On the basis of these experiments an increase in CO_2 tension in the capillaries of a muscle *in vivo* would be expected to cause a shift of potassium from muscle to blood comparable to the shift of chlorine from plasma to corpuscles.

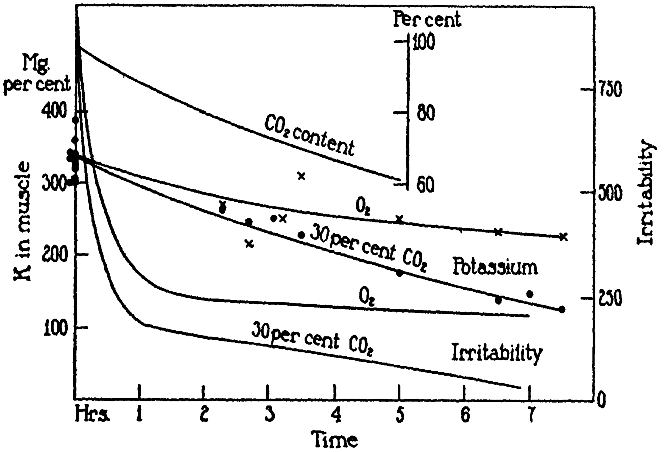

FIG. 6. The effects of equilibrating muscle plus Ringer's phosphate solution (pH 7.25) with 30 per cent CO_2 plus 70 per cent O_2. Decreases in potassium content, in mg. K per 100 gm. muscle, in irritability (ohms per volt in primary of inductorium), and combined CO_2 content (in per cent of the initial value) are plotted against the duration of immersion in the solution.

Some of these muscles were also put into respirometers after different periods of time, equilibrated with 30 per cent CO_2, and analyzed for combined CO_2 by dumping citric acid. The average initial content was 42.4 vol. per cent in six muscles. The decrease in HCO_3 with time is indicated in the uppermost graph in Fig. 6 in per cent of the initial value. The experimental error of the few figures obtained was sufficient to obscure any difference between the muscles equilibrated with 30 per cent CO_2 and the controls which were exposed to CO_2 only at the time of analysis. Both muscles were presumably slightly more alkaline than the solution and both showed a decrease to 60 per cent of the initial HCO_3 in 5 hours. Thus the potassium loss in 5 hours is 4.1 m.-eq. per 100 gm. while the CO_2 loss is 0.76 m.-eq. It is impossible to account for this large potassium loss without further analyses for Na, P, lactic acid, etc.

This sudden loss of irritability resembles at first thought the sudden loss of irritability described by Gellhorn (1930) when the concentration of potassium in the solution exceeds a certain critical value depending upon the amount of calcium present. We have tried, however, the concentrations of salts recommended by Gellhorn for the preservation of normal irritability and continue to find the same rapid initial drop in irritability. This drop is so rapid that one must work quickly in order not to miss it. Sometimes even a minute or two makes a big difference. The second muscle dissected of a pair of sartorius muscles usually had a lower irritability than the first. Semitendinosus and ileofibularis muscles give similar results. In Ringer's solution containing no potassium the initial fall in irritability is more rapid than in high potassium solutions. Possibly the immediate effect of potassium is to partially depolarize the membrane and increase irritability, while higher concentrations or more prolonged exposure may destroy the membrane and so abolish excitability. Lapicque and Nattan-Larrier (1926) have reported an initial decrease in chronaxie followed by an increase as a result of treatment with potassium. The injurious effect of both high and low potassium solutions are reversible to a certain extent.

Another surprise lies in the discovery that the irritability is increased by increase in the potassium content of the Ringer's solution up to a certain optimum. The optimum concentration of potassium according to our experiments is about 20 or 25 mg. per cent K, whereas usual Ringer's solution contains only about 5 mg. per cent K (0.01 per cent KCl). We at first thought that this optimum corresponded to the concentration at which the muscle was in potassium equilibrium, there being a decrease in irritability when potassium was either lost or gained by the muscle. The graphs of Fig. 7 show, however, that this is not the case because the point of optimum irritability does not vary significantly with the pH. Thus at pH 6.3 the muscle is in equilibrium (as to K) with a solution of 75 mg. per cent K whereas the optimum for irritability is at 20 mg. per cent. Where the points on these irritability curves are close enough together it is often possible to see that there is a very sudden drop after the concentration passes a certain critical value. One of the curves at pH 7.3 (Fig. 7) shows a suggestion of this effect. The drop is probably also sudden with respect to time and may correspond therefore to Gellhorn's sudden drop of irritability.

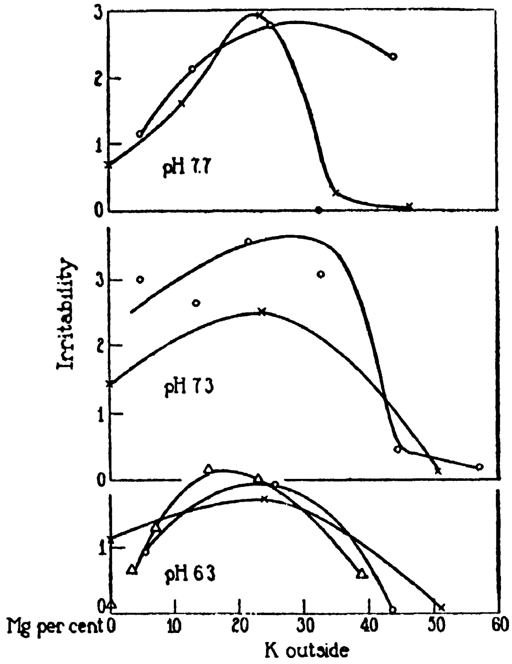

FIG. 7. Irritability (in ohms × 10^{-2} per volt in the primary of the inductorium) of muscles after 5 hour immersion in solutions of varying potassium content. Note that the optimum does not vary regularly with the pH. Each point represents one muscle.

We are quite well aware that this maximum of irritability at a relatively high potassium concentration may be due to our method of measuring irritability and we are not disposed to defend this simple induction coil method very far. In such experiments as we have tried, however, the chronaxie criterion gave a similar result. A similar optimum concentration of potassium was found by Sereni (1925) for tension and heat production but in this case the optimum coincided with the usual concentration in Ringer's solution.

Potassium Diffusion vs. Time

In most of our experiments a period of diffusion of 5 hours was arbitrarily selected as convenient. A few observations were made, however, at other durations so that the gain and loss of potassium as a function of time could be plotted. In each case the solutions were in equilibrium with 5 per cent CO_2 and the temperature was 22°C. Curves representing four such experiments are plotted in Fig. 8. In acid solutions, low in potassium, there is a steady loss of potassium from the beginning. In another equally acid solution (pH 6.3) the potassium concentration was high enough (51 mg. per cent) to cause an initial gain in potassium followed eventually by a loss. (Note one widely divergent point, a not uncommon result.) This subsequent loss may have been due to the increasing acidity inside and the destructive effect of high potassium upon the membrane. Even at neutrality and 30 mg. per cent K, however, the initial gain in potassium was not maintained. Only at pH 7.7 and 39 mg. per cent K did the experimental muscle continue to show a net gain 24 hours after the control muscle was analyzed. Some but not all of this gain might

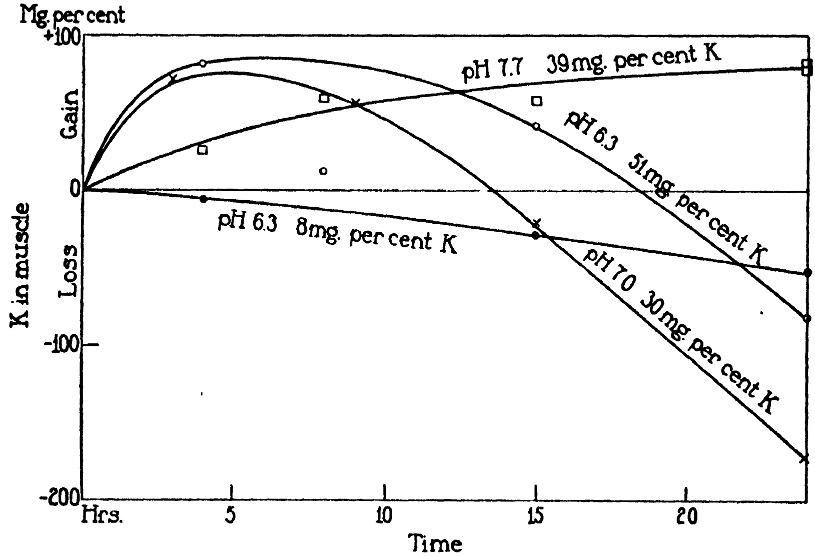

FIG. 8. Typical curves showing gain or loss of potassium by muscles as a function of the time of immersion in hours.

be due to an increase in the concentration of potassium in the tissue spaces until equal to that in the solution.

DISCUSSION

The evidence just presented shows clearly that potassium diffuses in and out of muscles by exchange with hydrogen ions or some equivalent process. Otherwise it is hard to explain how potassium can move against the concentration gradient after a slight increase in the concentration of potassium outside. Furthermore we have shown that potassium tends to move toward the side of increased acidity and when potassium is increased inside the muscle we have demonstrated an increase in bicarbonate content. The changes in bicarbonate, however, are not quantitatively sufficient nor are they always even in the right direction to explain completely the potassium movement. It is therefore evident that under the conditions of these experiments some anion or cation other than K must have diffused across the membrane. This fact is not in agreement with the general theory that the muscle membrane is anion-impermeable and permeable only to the smaller cations, K and H. To explain this conflict we are now attempting to determine what other ions are involved in this equilibrium shift. It is necessary to include in the study all chemical reactions within the muscle which can change the acid-base balance, *e.g.* the formation of lactic acid and the synthesis of phosphocreatine. Meanwhile we must consider the possibility that ions other than potassium can diffuse only outside the physiological range where the muscle has been somewhat injured or, that there is enough diffusion between solution and tissue spaces to account for the conflicts with the theory which have been observed. This explanation does not appear very hopeful to us but we are not prepared to rigidly exclude it until the total electrolyte balance is better explored.

There is, however, another experimental conflict with the theory which is still harder to exclude completely. This is the finding that the pH of the muscle is at least approximately the same both inside and out while the potassium concentration differs markedly. This might mean that the membrane is permeable to potassium but not to hydrogen ions. This, however, seems definitely contradicted by the evidence outlined above for a diffusion of potassium by exchange with hydrogen. Even a diffusion of potassium as undissociated KOH as suggested by Osterhout (1930) amounts to an exchange with hydrogen ions if water can penetrate. It has been argued that the membrane is impermeable to hydrogen ions because a change in pH does not affect the injury potential to the same extent that a change in potassium concentration does. It should be noted, however, that the absolute concentration of potassium ions is so large that the relatively infinitesimal number of hydrogen ions can have very little effect upon the phase boundary potential so that the argument is quantitatively fallacious.

Two different theories have been proposed by Osterhout (Osterhout (1930)); and Osterhout and Stanley (1932) and by Brooks (1929) to explain the accumulation of potassium in living cells. According to both theories, however, the potassium balance is not an equilibrium phenomenon at all but merely the result of a steady state of diffusion. The potassium accumulation is due then to a greater mobility of potassium as compared to sodium and to a continuous production of acid by CO_2 formation inside the cell. However well this scheme may work for plant cells it does not seem appropriate for muscles. In the first place the muscle cells are small and well ventilated by blood so that the CO_2 *tension* inside is very little greater than that outside and does not seem to be quantitatively sufficient to explain the great difference in potassium concentration. Further potassium cannot continue to enter a muscle cell as it can a *Valonia* cell for the muscle is not continuously growing and absorbing water. Without this provision the inequality in potassium concentration would not persist. Therefore it must be concluded that although such steady state theories may permit unequal K_o/K_i and H_o/H_i ratios the explanation is inadequate on other grounds.

It might be suggested that inequality between K_i/K_o and H_i/H_o ratios might be due to differences in the activity coefficients inside the cell as compared to those in solutions. Hill and Kupalov (1930), however, have shown that the osmotic pressures inside and outside the muscle are equal if all the potassium is assumed to be in solution so that one could hardly expect a change in activity coefficient of K_i sufficient to change this ratio very much. A change in activity of hydrogen ions could hardly explain a pH shift from the theoretical

5.52 to 7.0 and the change in the activity of HCO_3^- ions which Meyerhof, Möhle, and Schulz (1932) have found only makes matters worse.

While the theory of Mond and Netter appears to be incomplete it also fits the facts remarkably well in many ways. Without rejecting it altogether we prefer to suppose for the present that there is some independent mechanism within the muscle which regulates the pH to approximate neutrality in spite of the demands of the membrane equilibrium. Some continuous supply of energy would obviously be necessary for this purpose which might help to explain the resting oxygen consumption. This suggestion, however, is obviously of little real value until a detailed mechanism can be offered. We are, however, emphatically of the opinion that this equality of pH inside and out cannot be explained away by considerations of tissue spaces for example but must be included in the theory as an important item.

SUMMARY

1. Analyses were made of the K and HCO_3 content, the irritability, and weight change of isolated frog sartorius muscles after immersion for 5 hours in Ringer's solutions modified as to pH and potassium content.

2. At each pH a concentration of potassium in the solution was found which was in diffusion equilibrium with the potassium in the muscle. In greater concentrations potassium moved into the muscle against the concentration gradient and *vice versa.*

3. The greater the alkalinity of the solution the smaller the concentration of the potassium at equilibrium so that the product of the concentrations of OH and K in the solution at equilibrium tends to remain approximately constant.

4. The pH inside the muscle is approximately equal to that outside when first dissected but it tends to change during immersion so as to follow the changes in the pH of the solution. This finding is in direct conflict with the theory according to which the high potassium concentration inside should be accompanied by an equally high hydrogen ion concentration in relation to that outside.

5. The diffusion of potassium into the muscle makes its contents more alkaline but the increase in alkalinity is not always, nor usually, equivalent to the amount of potassium which has diffused and con-

versely, the pH inside can change in either direction according to the pH outside without there being any diffusion of potassium. Hence potassium is not the only penetrating ion.

6. The irritability of the muscles is at a maximum in concentrations of potassium which are greater than that in normal Ringer's solution, or about 20 mg. per cent potassium. This optimum does not seem to be a function of pH and is therefore not dependent upon the direction of movement of the potassium but probably on the ratio of potassium outside to that inside.

7. Swelling of the muscles occurs in solutions which injure the muscle so as to permit both cations and anions to enter without permitting the organic protein anions to escape. Anion impermeability is necessary to prevent this same osmotic swelling under normal conditions.

8. An increase in the CO_2 tension in muscle and solution causes a greater increase in acidity in the solution than in the muscle and leads to a loss of potassium. One expects therefore a potassium shift from tissues to blood comparable to the chlorine shift from plasma to corpuscles.

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THE DIFFUSION OF CARBON DIOXIDE IN TISSUES

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The diffusion of CO_2 in animal membranes has received the attention of but few investigators and the data presented have been inadequate and apparently contradictory. Krogh (1919) published a permeability constant for CO_2 in connective tissue based on a single determination. He considered his value too high although it is lower than a similar constant given by Hagenbach (1898) for CO_2 diffusing in 20 per cent gelatin. Fenn (1928 *b*) published diffusion coefficients for CO_2 in nerve and muscle and found values considerably lower than the diffusion coefficient that he calculated from Krogh's permeability constant. Considering the contradictory results obtained by the above authors and the fundamental importance of the knowledge of the rate of diffusion of CO_2 in physiological problems it seemed desirable to make a study of the diffusion of this gas in different types of membranes. Two methods of determining the rate of diffusion of CO_2 in tissues and the results of measurements made on connective tissue, muscle, and frog skin are given below.

Apparatus and Methods

Measurement of Permeability.—The method of measuring permeability of tissues to CO_2 depends on the fact that small amounts of CO_2 can be detected by conductivity changes in a barium hydroxide solution (Fenn, 1928 *a*). Essentially the method is to pass CO_2 of a known tension over one face of a tissue and catch the CO_2 that leaves the opposite face in barium hydroxide. Measurements of the conductivity of the barium solution are made by means of a suitable Wheatstone bridge and the changes are expressed in cc. of CO_2 at standard pressure and temperature.

The apparatus used is shown in Fig. 1. It consists of a conductivity cell with two platinum electrodes sealed into the side arm of a bottle, fitted with a ground glass stopper. The brass tube (*A*) is sealed into the glass stopper with de Khotinsky cement. Two brass rings (*B*) are used to hold the tissue and these fit snugly over the lip at the base of the brass tube (*C*). When the tissue is in place, the tube (*C*) is inserted into tube (*A*) and the tissue is trapped under the lip of tube (*C*), leaving an area of 0.317 cm.[2] exposed for diffusion. The gas is saturated with water at the experimental temperature (22°C.) and then enters the diffusion chamber through the glass tube (*D*). The side arm with the stop-cock is open until the apparatus has come to temperature equilibrium with the water bath in which the whole apparatus is immersed.

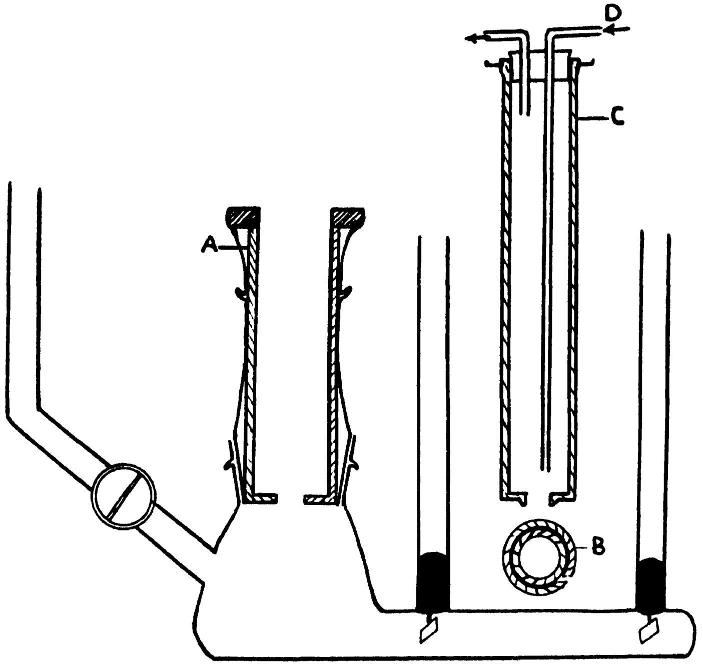

FIG. 1. Apparatus for measuring the permeability of tissues to carbon dioxide.

The values given by Fenn for the change in conductivity per c. mm. of CO_2 at different specific conductivities of barium hydroxide have been (1) multiplied by 0.95 to reduce them from 25°C. to 22°C.; (2) multiplied by $\frac{9.28}{9.75}$ to correct for the difference in cell constants; (3) the ordinates have been multiplied by $\frac{3.97}{6.00}$ to correct for the volume of barium hydroxide used.

The routine procedure for an experiment is to prepare the tissue in the rings, wash well with Ringer's solution, make several determinations of its thickness, and then immediately mount it in the conductivity cell containing a known quantity (6.0 cc.) of barium hydroxide solution. The small space between the two brass tubes is well sealed with wax and the cell is immersed in a constant temperature bath. The gas, previously mixed in two 20 liter bottles, is then passed slowly through a saturator (also in bath) before entering the diffusion chamber and the whole apparatus is allowed to stand until an equilibrium is established. 20 minutes were found to be sufficient to flush out all parts with the CO_2 mixture and to arrive at the bath temperature. The conductivity of the barium hydrate is now measured and subsequent readings taken at intervals until the rate of passage of CO_2 has remained constant for a period of 2 hours. The results are expressed in cc. of gas (N.T.P.) passing through 1 cm.^{2} under a pressure gradient of one atmosphere per cm.

Measurement of Absorption Coefficients.—According to Fick's law of diffusion, the quantity of gas (ds) which passes in a time (dt) across an area (A) under a concentration gradient $\frac{(dc)}{(dx)}$ is given by the formula

$$ds = KA\frac{dc}{dx}dt \tag{1}$$

where (K) is the true diffusion coefficient for the substance. With appropriate units K has the dimensions $\frac{\text{cm.}^2}{\text{minute}}$. In order to derive a diffusion coefficient from the permeability constant $\left(\frac{\text{cm.}^2}{\text{minute atmosphere}}\right)$ as determined by the barium hydroxide method it is necessary to know the absorption coefficient $\left(\frac{\text{cm.}^3}{\text{cm.}^3\text{ atmosphere}}\right)$ for CO_2 in the tissues. Dividing the permeability constant by the absorption coefficient the true diffusion coefficient $\left(\frac{\text{cm.}^2}{\text{minute}}\right)$ is obtained.

A differential volumeter of the type used by Fenn (1927) was modified as shown in Fig. 2 for the determination of the absorption coefficient. The experimental bottle (A) has a side pocket (B) large enough to contain 2.5 cc. of mercury and a side arm with a stop-cock (C) for flushing the bottle with gas. A ground glass stopper fits into the base of the experimental bottle and during an experiment contains a flat piece of acidified tissue stretched over a brass disc and held in place by a split ring. The volume of the tissue is determined from the weight (specific gravity 1.04) and the thickness determined by the method described below. The whole apparatus is immersed in a water bath at $22°\pm0.01°C$.

The tissue is weighed and placed in the stopper. The apparatus is then placed in the water bath and the experimental bottle flushed out with hydrogen, saturated with water at 22°C. The stop-cocks are turned to the position connecting both bottles with the capillary and an equilibrium established. At this stage the tissue is saturated with hydrogen and mercury is tipped from the side pocket, trapping the hydrogen in the tissue. The stop-cocks are now turned to permit flushing of the bottle with CO_2. 5 minutes are allowed for thorough washing out of all hydrogen and then the cocks are turned again to the capillary. When equilibrium is again reached (2 to 3 minutes) the mercury is quickly dumped into the side pocket and CO$_2$ is exchanged for the hydrogen in the tissue, resulting in a rapid movement of the kerosene drop toward the experimental bottle. When the drop in the capillary again comes to equilibrium the tissue has become saturated with

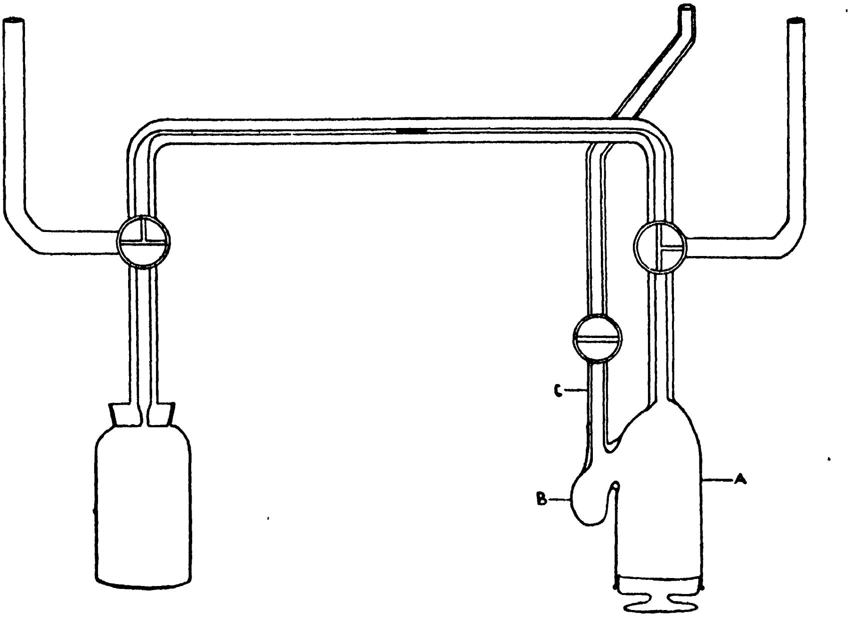

FIG. 2. Apparatus for the determination of the carbon dioxide absorption and diffusion coefficients for tissues.

CO$_2$ and the amount required for saturation can be calculated by means of the following formula from Fenn (1928 *a*):

$$x = d\frac{V_c + V_r}{V_c + d}\,\frac{(P - y)}{P} \qquad (2)$$

where

x = volume of CO$_2$ (temperature and pressure of experiment) required for saturation;

d = volume of capillary (8.6×10^{-3} cc. per cm.) per unit length;

V_c = volume of control bottle (19.4 cc.);

V_r = volume of experimental bottle (30.3 cc.);

P = barometric pressure;

y = vapor pressure in the bottles.

The value x is corrected to standard temperature and pressure in these experiments.

Some determinations of the amount of CO_2 leaving the tissue have been made by covering the CO_2 saturated tissue with mercury and flushing the bottle with hydrogen. When the mercury is dumped into the side pocket the CO_2 escapes and the kerosene drop moves away from the bottle in proportion to the amount of CO_2 dissolved.

A correction must be made for the hydrogen leaving the tissue when calculating the amount of CO_2 necessary for saturation. This correction is only small due to the low solubility coefficient for hydrogen in H_2O (0.018 cc./cc.). A further correction is made for any constant drift of the kerosene drop previous to dumping the mercury and after saturation. Such a correction, while not large, becomes significant over a period of 20 or more minutes. The maximum rate of movement noted was 0.04 cm./minute which in 20 minutes amounts to about 5 per cent of the total movement. The average drift was 0.02 cm./minute.

Measurement of the Diffusion Coefficient by the Volumetric Method.—In the experiments for determination of the absorption coefficient, we have a condition where a flat sheet (of known thickness) is suddenly exposed to a gas and the percentage saturation at any time thereafter is indicated by the position of the kerosene drop. Under such conditions a diffusion coefficient for the gas in the material of the sheet can be calculated by the formula

$$\frac{Q}{Q_1} = 1 - \frac{8}{\pi^2}\left\{e^{-\frac{\pi^2}{4}.\frac{Kt}{a^2}} + \frac{1}{9}e^{-\frac{9\pi^2}{4}.\frac{Kt}{a^2}} + \frac{1}{25}e^{-\frac{25\pi^2}{4}.\frac{Kt}{a^2}} + \ldots.\right\} \tag{3}$$

taken from the work of Andrews and Johnston (1924).

Q_1 is the total amount of the gas present in the sheet at saturation. Q is the amount present at the time t. a is the thickness of the tissue and K the diffusion coefficient of the gas.

This equation can be reduced to the simpler form

$$\frac{Kt}{a^2} = -0.0851 - 0.933 \log\left(1 - \frac{Q}{Q_1}\right) \tag{4}$$

if all terms but the first are neglected, a condition sufficiently accurate for present purposes. When $\frac{Kt}{a^2}$ is greater than 0.1, corresponding to 36 per cent saturation, the error is less than 1 per cent.

When the mercury is tipped off the tissue, during a determination of the absorption coefficient, a stop-watch is started and the position of the kerosene drop read at intervals of 30 seconds for the first 2 minutes, and appropriate intervals thereafter, until equilibrium; *i.e.*, saturation is reached. From the position of the drop, at any time after tipping, the percentage saturation can be determined and K can be readily calculated from Equation 4 when a is known. The position of the drop must be corrected for the H_2 diffusing from the tissue as well as any constant drift as mentioned above. The rate at which H_2 leaves the tissue may be calculated by means of Equation 4, assuming a diffusion coefficient of 28.0 ×

10^{-4}. This figure is obtained by assuming that the rate of diffusion is inversely proportional to the square root of the gas density and the coefficient of diffusion for CO_2 is 6.0×10^{-4}. A number of determinations of the CO_2 diffusion coefficient in acidified tissues have been made by this method.

The CO_2 used in all experiments was taken from cylinders as supplied by the Ohio Chemical Company for medicinal purposes. This CO_2 is pure within a small fraction of 1 per cent by analysis. All CO_2 mixtures above 30 per cent were analyzed by flushing out a capillary tube (with stop-cock) and allowing the open end to stand in NaOH. The ratio of the height to which the NaOH rises in the tube to the total length of the tube gives the percentage CO_2 in the gas mixture within 1 per cent. All CO_2 mixtures below 30 per cent were analyzed on the Haldane-Henderson gas analyzer.

Measurement of Membrane Thickness.—Probably the greatest source of error in all permeability and diffusion measurements lies in the determination of the thickness of the membrane used. Many preliminary experiments in this work have been rejected due to variability, traceable directly to errors in the methods of measuring thickness. The apparatus used in all the experiments below is shown in Fig. 3. (*A*) represents the objective of a microscope fitted with a tapered metal pointer and (*B*) a brass plate with an elevated disc resting on the stage of a microscope. An electric circuit is completed from the pointer to the plate through a rheostat and a pair of head-phones. An oscillating current is furnished by the vacuum tube oscillator (*C*). The tissue, already in the rings (*D*), is placed over the elevation on the brass plate and the objective is lowered by means of the fine adjustment screw (calibrated) until the pointer just makes contact as signified by the hum in the phones. By taking alternate readings with the 3 gm. weight (*E*) on the tissue and directly on the platform, a series of figures is obtained the average of which gives an accurate determination of the thickness. For example, three thickness determinations on the same membrane gave the following values: 0.235 mm., 0.238 mm., and 0.230 mm., each an average of six to ten measurements. The deviation of any single reading from the mean of ten readings seldom exceeds 5 per cent.

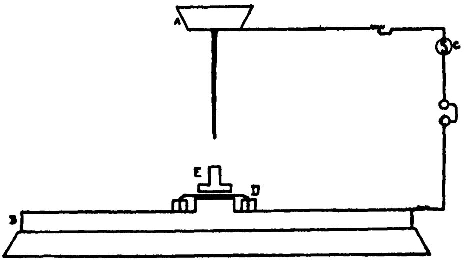

FIG. 3. Apparatus for measuring the thickness of tissues.

RESULTS

Permeability Constant.—Fig. 4 shows the graph of a typical experiment using the barium hydroxide method for the determination of the permeability of tissues for CO_2. This graph shows the constancy of the rate at which CO_2 passes through a tissue after an initial period required to reach equilibrium.

Table I contains a summary of all the determinations of membrane permeability obtained by the barium hydroxide method. The average thickness of the tissue is given, together with the extreme range. The tissues decreased in thickness during the period of the determination. This decrease was, on the average, 10 per cent of the total thickness. The fourth column shows the extreme range of CO_2 tensions used and the last column the permeability constant together with the probable error.

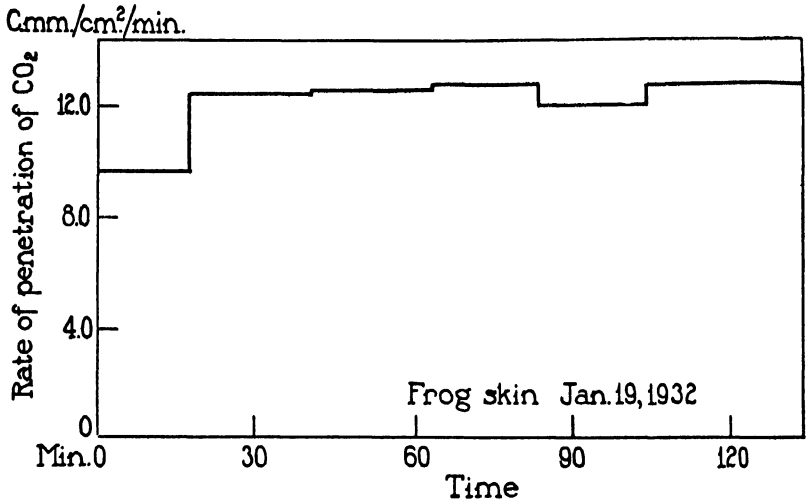

FIG. 4. Graph showing constancy of the rate at which carbon dioxide penetrates frog skin. Thickness 0.237 mm.; CO_2 tension 734 mm. Hg; temperature 22°C.

The three determinations on the permeability of rubber were made on membranes cut from the same sheet. Rubber was chosen as a means of checking the reliability of the method by comparison with the permeability as determined by other workers. The individual determinations gave the following values: 0.47, 0.50, and 0.47×10^{-4}

which are in good agreement with the permeability constant 0.44×10^{-4} as found by Daynes (1920). The determinations on rubber also show the variation to be expected using a membrane of constant properties.

Twenty-five determinations on frog skin, cut from the belly of the frog, gave an average permeability constant of 3.05×10^{-4} with a probable error as shown. Acidification (4 or more hours in N/10 HCl) of frog skin markedly reduces the resistance offered to the passage of carbon dioxide as can be seen from the average value of 4.47×10^{-4}. This change may be attributed to several factors such as sloughing off of the outer epithelial layer, a 5 to 10 per cent increase in water content, and general structural changes accompanying precipitation of proteins.

TABLE I

Permeability Constants for CO_2 Diffusing in Various Media

	No. of determinations	Average membrane thickness	Range of CO_2 tension	Permeability $(P) \times 10^4$
		micra	*mm. Hg*	
Rubber	3	50	84–86	0.48
Frog skin	25	248 (175–325)	57–744	$3.05^{\pm 0.23}$
*Frog skin (acidified N/10 HCl)	4	283 (270–300)	722–734	$4.47^{\pm 0.01}$
Connective tissue (dog diaphragm)	2	215 (175–255)	127–148	2.65
Frog muscle	26	354 (255–500)	74–731	$5.29^{\pm 0.54}$
Mammalian muscle (dog diaphragm)	1	700	152	†4.7
Smooth muscle (cat bladder)	1	360	109	†5.0
Parchment paper	2	233	728–732	1.35
‡Parchment paper ($NaHCO_3$)	2	235	727–730	1.54

Permeability is expressed as cc. per $cm.^2$ per min. with a gradient of one atmosphere per cm. $\times 10^4$.

Figures in parentheses in Column 3 indicate the extreme ranges of thickness of tissues.

* Frog skin soaked for 4 or more hours in N/10 HCl.

† Corrected for connective tissue.

‡ Parchment soaked for 12 hours in 0.15 M $NaHCO_3$.

All experiments at $22° ^{\pm 0.01}$°C.

Mammalian connective tissue offers slightly more resistance to the passage of CO_2 than does frog skin. These membranes were taken from the central tendon of the diaphragms of two dogs. The permeability was 2.65×10^{-4}.

Muscles, both of frogs and mammals, are more freely permeable to CO_2 than connective tissue or frog skin. Twenty-six determinations, on the combined externus and internus obliquus muscles from the abdominal wall of the frog, gave an average value of 5.29×10^{-4}, uncorrected for the thin layers of connective tissue. A correction for connective tissue, estimated at 5 per cent of the total thickness, gives a value of 5.4×10^{-4}. The permeability seems to vary somewhat with the season, being somewhat lower in the winter months. This difference, however, may be due only to the difference in batches of frogs used. The single determinations on the diffusion through mammalian striated and smooth muscle are not greatly different from the average value for frog muscle. The values for mammalian muscles have both been corrected for the layers of connective tissue. The thickness of these layers (measured) was equal to 15 per cent of the total thickness and the correction made on the basis of the permeability measurements given for connective tissue in Table I. Allowance for connective tissue raises the permeability constant from 4.4×10^{-4} to 4.7×10^{-4} as given.

The frog muscles were still slightly irritable at the end of a determination and the frog skin appeared to be in good condition. The mammalian tissues were used directly after removal from the animal and were definitely not "normal" after 2 to 4 hours at 22°C. However, there was no marked change in permeability over this period.

The amount of CO_2 produced by metabolism in the tissue has been neglected in the calculation of the above constants, since it forms as a maximum only 1 part in 100 of the total gas diffusing.

Absorption Coefficient.—Diffusion rates expressed as a permeability constant (P) are useful when considering the diffusion of gases in a steady state; *i.e.*, when equilibrium has been established and the concentration gradient remains constant. When dealing with the kinetics of diffusion, however, Fick's diffusion coefficient ($K = P/\alpha$), as given above, must be known. The determination of the absorption coefficient (α) presents some difficulty since CO_2 is present both in the physically dissolved and in the bicarbonate form. If the bicarbonate ion assists in the diffusion of CO_2 in the steady state then the diffusion coefficient for total CO_2 will be $K = \frac{P}{\alpha + B}$ where B is a modifying factor due to the presence of CO_2 in the form of bicarbonate. If the bicarbonate ion does not diffuse appreciably we can proceed as though it were not present except for the slight salt effect which it may have on the solubility of CO_2. The following evidence indicates that the diffusion of bicarbonate is negligible when determining permeability.

In order that the permeability (*P*) should remain constant at different experimental gas tensions, the amount of diffusable CO_2 dissolved must be a linear function of the gas tension. That the total CO_2 absorbed by tissues is not proportional to the tension is evident from the CO_2 dissociation curves of muscle and nerve as determined by Fenn (1928 *b*). Above 50 mm. Hg tension of CO_2, the amount of combined CO_2 (bicarbonate) is relatively constant. Assuming that bicarbonate diffused alone, the gradient or diffusion pressure would be practically the same for tensions of CO_2 above 50 mm. of Hg. This would mean that approximately equal amounts of CO_2 would be transported over a wide range of experimental gas tensions and the permeability constant would vary with the tension used. Inasmuch as the permeability is found to be constant and independent of tension, it might be inferred that bicarbonate contributes but little to the total diffusion.

The experiments on the permeability of parchment paper (Table I) give a further indication of the small part played by bicarbonate in the total carbon dioxide transport. The first two determinations on parchment soaked in Ringer's solution (bicarbonate free) gave permeability constants 1.34 and 1.36×10^{-4}. The two determinations on the same membrane soaked in 0.15 M bicarbonate solution gave the permeability constants 1.52 and 1.55×10^{-4}, an increase of about 12 per cent. If such a concentration of bicarbonate increases the rate of diffusion only by 12 per cent, it seems reasonable to assume that in tissues where the bicarbonate concentration is but one-tenth as great (approximately 0.015 M), bicarbonate would contribute a negligible amount to the total diffusion.

It was thought at first that acidification of the tissue would solve this difficulty by removing the bicarbonate. However, such a procedure (Table I) so lowers the resistance offered by the tissue that diffusion is even faster with only dissolved CO_2 present.

Since bicarbonate contributes so little to the total diffusion, only the physically dissolved CO_2 has been considered in the determination of the absorption coefficient. This amount may be estimated, assuming that CO_2 dissolves in the tissue as though it were 80 to 83 per cent water and the solids were inert. The carbon dioxide absorption coefficient for water at 22°C. is 0.829 cc. (Landolt-Börnstein tables). On this assumption the tissues would take up 0.67 cc. per cc. at 22°C. under a pressure of one atmosphere.

The work of Van Slyke (1928) on the solubility of CO_2 in acidified blood corpuscles shows that the organic constituents of tissue are not inert but dissolve CO_2. On the basis of Van Slyke's figure of 0.45 cc. for the absorption coefficient of CO_2 in acidified ox red blood corpuscles (71.7 per cent H_2O) at 37°C. an estimate can be made of the solubility in acidified muscle or other tissue at 22°C. Assuming that the temperature coefficient for CO_2 solubility in tissues is the same as for water (-0.017 cc. per degree) and correcting for the difference in water content, one arrives at the figure 0.77 for the solubility coefficient of CO_2 in acidified tissues. This value is higher than the solubility (0.67 cc. per cc.) as calculated from the water content.

Van Slyke was unable to analyze for CO_2 on acid solutions containing more than 25 per cent cells due to the viscosity of the solution. His value of 0.45 cc. for the solubility in 1 cc. of cells was obtained by extrapolation from 25 per cent to 100 per cent cells. A determination of the solubility of CO_2 in tissues based on his extrapolated figure and requiring correction for temperature and water content is rather indirect. It was, therefore, thought advisable to make some actual determinations of the absorption coefficients for CO_2 in the tissues used. The apparatus and procedure used for this purpose are given above.

Table II, Column 2, shows the results of the solubility determinations made on frog skin and muscle acidified with N/10 HCl. The average value 0.78 cc. per cc. for the absorption coefficient for CO_2 in frog skin agrees almost exactly with the value calculated from Van Slyke's data on acidified red blood corpuscles and is somewhat lower than the coefficient of solubility for CO_2 in water (0.829 cc. at 22°C.) as given in the Landolt-Börnstein tables. The average value for the solubility of CO_2 in acidified muscle is somewhat higher (0.84 cc. per cc.). The water content of the frog skins increased on an average 6 per cent, due to acidification, so that the value for the solubility of CO_2

in normal tissue is probably somewhat less, 0.73 cc. per cc. for frog skin. A similar correction for increased water content of muscle (average increase 8 per cent) gives a value 0.78 cc. per cc. for the solubility of CO_2 in normal muscle. The corrected values have been used in all calculations below.

TABLE II

The Absorption Coefficients and Diffusion Coefficients for CO_2 in Acidified Tissues, as Determined by the Volumetric Method

Tissue	No. of determinations	Absorption coefficient	Determinations	Diffusion coefficient
Acidified frog skin	11	$0.78^{\pm 0.022}$	9	$6.7^{\pm 0.23}$
Acidified muscle	10	$0.84^{\pm 0.024}$	7	$6.4^{\pm 0.32}$

All determinations at 22°C.
Probable error is given.

TABLE III

Averages of Carbon Dioxide Diffusion Coefficients Determined by the Barium Hydroxide Method

Membrane	No. of determinations	Permeability constant	Absorption coefficient	Diffusion coefficient
		$\times 10^4$	*cc./cc.*	*cm.²/min.* $\times 10^4$
*Rubber	3	0.48	0.93	0.51
Frog skin	25	3.05	0.73	4.18
Frog skin (acidified)	4	4.47	0.78	5.7
Frog muscle	26	5.29	0.78	6.8
Muscle (dog)	1	4.7	0.78	6.0
Smooth muscle (cat)	1	5.0	0.78	6.4
Connective tissue (dog)	2	2.65	0.73	3.6

Permeability expressed as cc. per cm.² per min. under a gradient of one atmosphere per cm. × 10^4.

* Absorption coefficient based on Wroblewski's equation for the solubility of CO_2 in rubber, as given by Glazebrook.

Diffusion Coefficient.—Table III contains the averages of the CO_2 permeability constants as determined by the barium hydroxide method, together with the CO_2 diffusion coefficients as calculated by means of the solubility coefficients of Table II. The absorption coefficient for rubber has been calculated from Wroblewski's equation (see Glazebrook) for the solubility in water-saturated rubber ($\alpha = 1.2779 - 0.01576t$). The absorption coefficient for connective tissue has been arbitrarily taken as equal to that of frog skin.

The figures given for the diffusion coefficient indicate that all types of muscle offer approximately the same resistance to the diffusion of CO_2. On the other hand this gas is very much retarded when passing through connective tissue or frog skin, the diffusion coefficient for these tissues being only 60 per cent of that for muscle. The explanation for this difference must lie in a difference in structure of the tissues. For instance the muscle probably has a greater percentage of lymph interspaces than skin or connective tissue, and diffusion in such spaces should be as fast as in water (Table IV).

TABLE IV

The Diffusion of Carbon Dioxide in Various Media

Medium	Temperature	Permeability constant	Absorption coefficient	Diffusion coefficient	
	°C.	$\times 10^4$	*cc./cc.*	$cm.^2/min. \times 10^4$	
Water	16	9.4	0.99	9.5	Hüfner, 1897
Rubber	17	0.44	0.86	0.51	Daynes, 1920
Rubber	22	0.48	0.93	0.51	Wright
Gelatin 20 per cent	15	5.9	1.0	5.9	Hagenbach, 1898
Connective tissue (frog)	20	4.0	0.73	5.5	Krogh, 1919
Connective tissue (dog)	22	2.7	0.73	3.7	Wright
Muscle (frog)	22	0.85	0.78	1.17	Fenn, 1928
Muscle (frog)	22	5.3	0.78	6.8	Wright
Muscle (dog)	22	4.7	0.78	6.0	Wright
Smooth muscle (cat)	22	5.0	0.78	6.4	Wright
Nerve (frog)	22	0.55	0.78	0.71	Fenn, 1928
Frog skin	22	3.1	0.73	4.2	Wright

Permeability is expressed in cc. per $cm.^2$ per min. under a pressure gradient of one atmosphere per cm. $\times 10^4$.

The coefficient of Daynes has been multiplied by 60 and the coefficients of Hüfner and Hagenbach multiplied by $\frac{1}{1440}$ to change the units to minutes.

Krogh's value has been divided by and Fenn's figure multiplied by the proper absorption coefficients to complete the table.

In this connection it should be mentioned that the term diffusion coefficient is here applied to a membrane of tissue composed of many layers, involving different absorption coefficients and diffusion coefficients (Osterhout, 1933). However, the fact that the rate of saturation (Fig. 6) so nearly follows the theoretical based on an equation developed for an homogeneous medium, warrants the use of the term until more is known concerning diffusion in each layer.

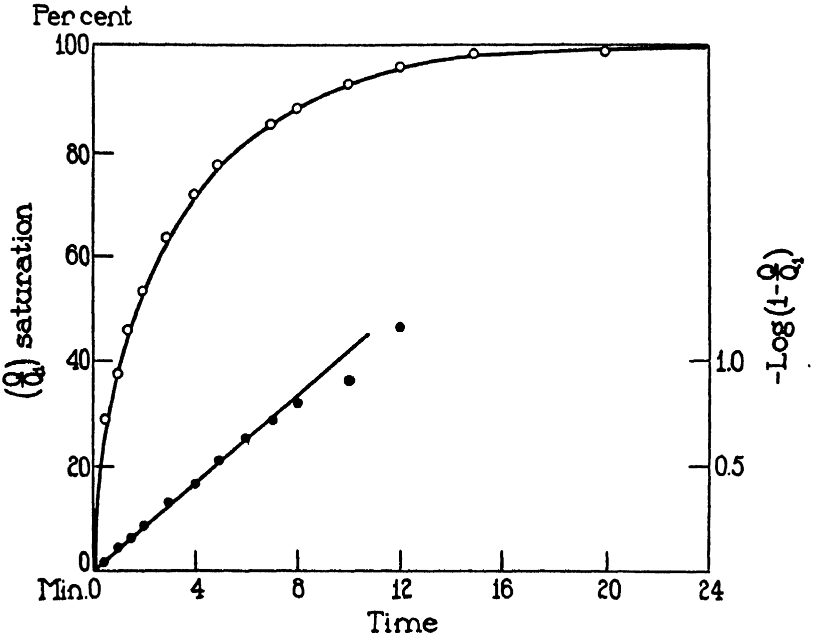

FIG. 5. Circles show the rate of saturation of a sheet of acidified frog skin with CO_2. Curve shows the theoretical rate of saturation when the diffusion coefficient is 6.8×10^{-4}. Lower line shows the relation between $-\log (1 - Q/Q_1)$ and t in Equation 4.

Diffusion Coefficients Determined by Volumetric Method.—Fig. 5 shows the graph of a typical experiment in which a piece of acidified frog skin saturated with hydrogen, was suddenly exposed to an atmosphere of CO_2 in a volumeter. The circles show the rate of saturation as indicated by the movement of the kerosene drop. The theoretical rate of saturation at different times as determined by substitution of 6.8×10^{-4} for K in Formula 4 above is shown by the curve. The experimental values follow closely the theoretical. The points on the lower line represent $-\log (1 - Q/Q_1)$ plotted against time and they fall regularly about a straight line up to 8 minutes, corresponding to 90 per cent saturation. The slope of this line is an average value for $\frac{-\log (1 - Q/Q_1)}{t}$ which when substituted in Equation 4 gives the value 6.8×10^{-4} for K, the tissue having a thickness of 0.083 cm.

From Formula 4 it is seen that all values of Q/Q_1 (per cent saturation) when plotted against t/a^2 should fall on a single curve. Fig. 6 shows a graph of all the experimental points obtained on acidified frog skin and muscle together with the theoretical curve for $K = 6.5 \times 10^{-4}$. The points fall regularly about the theoretical curve up to 90 per cent saturation. The last 10 per cent of saturation lags behind the theoretical rate. The reasons for this probably are (1) that the tissues were not entirely uniform in thickness over the total area and (2) that a small fraction of the tissue was held between a brass disc and a split ring approximately doubling the effective thickness at the edges.

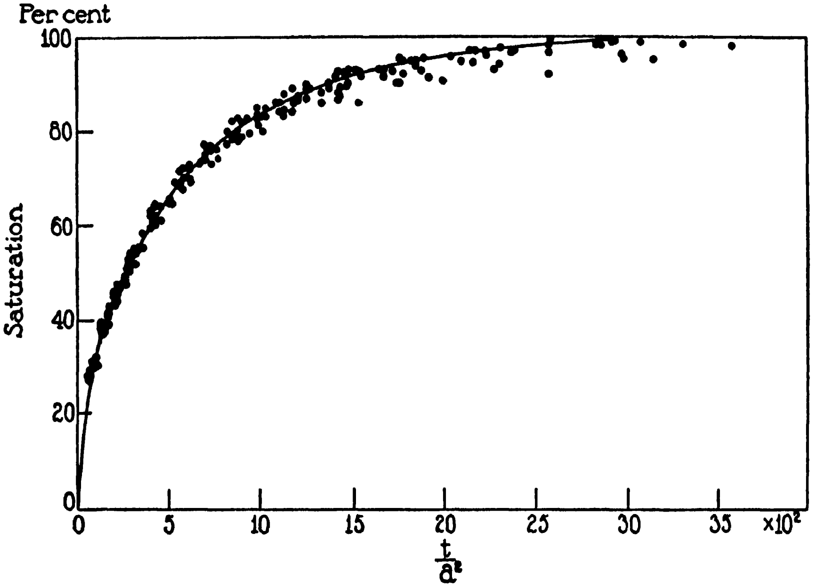

FIG. 6. Graph shows all the experimental points (per cent saturation) obtained by the volumetric method on acidified frog skin and muscle, plotted against $t/a^2 \times 10^2$. The curve shows the theoretical saturation when the diffusion coefficient is 6.5×10^{-4} at all values of $t/a^2 \times 10^2$.

A summary of the diffusion coefficients obtained with acidified tissues by the volumetric method is given in Table II. The average coefficient for frog skin is 6.7×10^{-4} and for muscle 6.4×10^{-4} with the probable error as shown. The value for frog skin can be directly compared with the diffusion coefficient (5.7×10^{-4}) as obtained on acidified skin by the barium hydrate method (Table IV). The agreement must be considered good. Larger variations in the determination of the thickness of a relatively large area of tissue when stretched over a brass plate together with the fact that fewer volumetric determinations were made lead the author to believe that the value 5.7×10^{-4} is the more exact. Acidified muscle tears and splits very easily and a satisfactory determination of its permeability has not been possible.

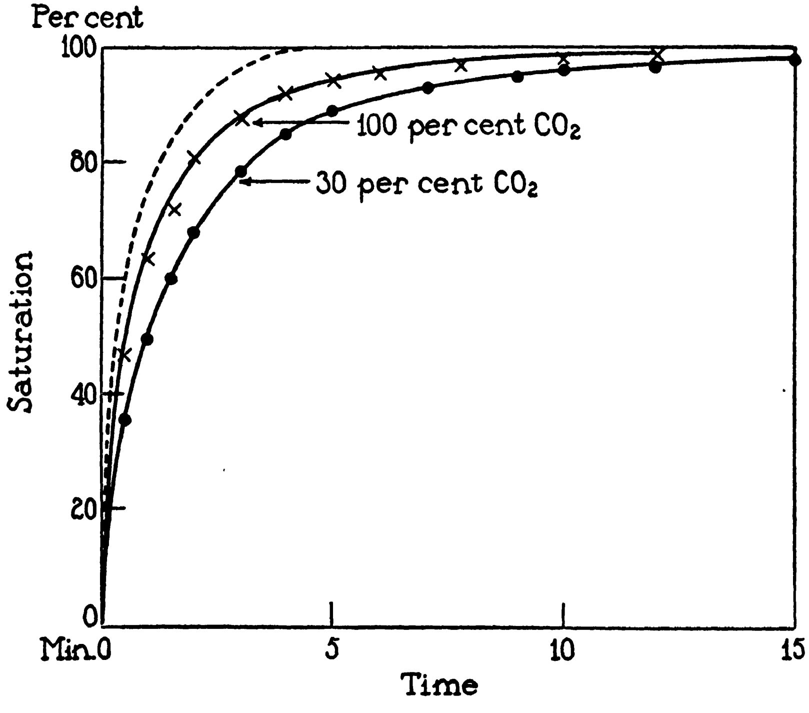

FIG. 7. Graph showing the different rates of saturation of non-acidified muscle (0.380 mm. thick) when exposed to different tensions of CO_2. The lower curves show the rate of saturation in atmospheres of 30 per cent and 100 per cent CO_2 as indicated. The broken curve shows the theoretical rate of saturation ($K = 6.0 \times 10^{-4}$).

The Rate of Saturation of Non-Acidified Tissues

When base is available for the formation of bicarbonate the rate of saturation of a tissue with CO_2 is definitely changed. This is shown in Fig. 7 where the rate of saturation of a disc of frog muscle (0.380 mm. thick) is plotted against time in minutes. The two lower curves show the experimentally determined rates of saturation of the same tissue at the CO_2 tensions indicated. The upper curve shows the theoretical rate of saturation if bicarbonate were not present. Fig. 8 is a similar graph for a double layer frog skin (0.610 mm. thick). The curves given are typical of twenty-two determinations on muscle and eleven determinations on frog skin. The rate of saturation is slower in the presence of bicarbonate and furthermore is dependent on the CO_2 tension to which the tissue is exposed, being slower at 220 mm. Hg than at 730 mm. Hg CO_2 tension.

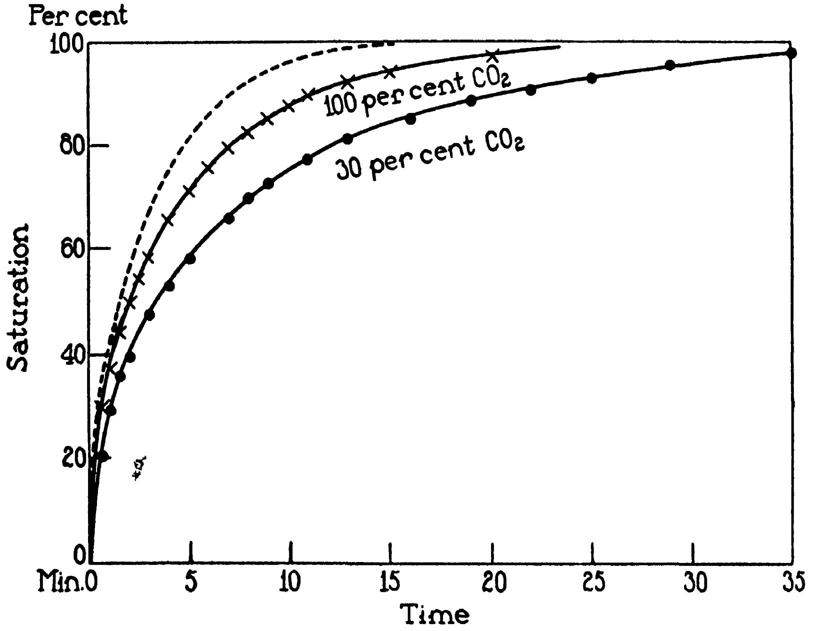

FIG. 8. Graph showing the different rates of saturation of a double layer of non-acidified frog skin (0.610 mm. thick). The lower curves show the rates of saturation at the CO_2 tensions indicated and the broken curve the theoretical rate of saturation ($K = 4.1 \times 10^{-4}$) if bicarbonate were not present.

It has been assumed that saturation is complete when the kerosene drop of the volumeter has reached a constant rate of movement. After saturation (20 to 30 minutes) the drop moves uniformly toward the experimental bottle at an average of 1.3 mm. per minute, representing a CO_2 absorption of 12 c.mm. per gm. per minute. This is more than five times the maximum rate of absorption due to phosphocreatine hydrolysis when sartorius muscles are exposed to an atmosphere of CO_2 (Lipmann and Meyerhof, 1930).

It should also be mentioned that after allowing for the large drift the amount of bound CO_2 (total minus H_2CO_3) at saturation is approximately twice as great as that found by Fenn (1928*b*) and Root (1933) at tensions of 70 mm. and 220 mm. Hg. The only apparent reason for this discrepancy is that the tissues were stretched tightly over a brass disc and in the work quoted they were at rest.

DISCUSSION

Table IV contains a collection of diffusion coefficients for CO_2 in various media. CO_2 diffuses in muscle approximately 65 per cent as rapidly as in water. In connective tissue and frog skin the diffusivity is even slower, about 40 per cent of that in water. The diffusion coefficient for gelatin is very nearly that for muscle. Krogh's value for the permeability of connective tissue is somewhat high as he judged. Fenn's "approximate" values for the diffusion coefficients for CO_2 in muscle and nerve are too low. They were calculated from the experimentally determined rate of saturation by means of Formula 4 given above. This formula does not apply when a chemical reaction takes place to change the rate of diffusion, in this case CO_2 to bicarbonate.

From Graham's law, that diffusion is inversely proportional to the square root of the molecular density, O_2 should diffuse 1.18 times as fast as CO_2. This law holds for these gases diffusing in water and is approximately true for diffusion in connective tissue. However, Krogh's value (1919) for the diffusion coefficient for O_2 ($K = 4.5 \times 10^{-4}$) is less than the diffusion coefficient for CO_2 (6.8×10^{-4}) in frog muscle. This is not a unique exception to Graham's law since Daynes (1920) found that H_2 diffused in rubber much more rapidly than would be expected in comparison with other gases.

It is a pleasure to acknowledge my indebtedness to Professor W. O. Fenn who suggested this problem and assisted me with advice and encouragement throughout the progress of the work.

SUMMARY

1. Two methods are given for measuring the rate of diffusion of CO_2 in tissue membranes. Methods are also given for the determination of tissue thickness and the absorption coefficient for CO_2 in tissues.

2. The values obtained for the permeability constant ($P \times 10^4$) at 22°C. for CO_2 in the following tissues are:—frog skin, 3.05; connective tissue (dog), 2.65; smooth muscle (cat), 5.00; frog muscle, 5.29; striated muscle (dog), 4.70. P is expressed as cc. per cm.^{2} per minute under a pressure gradient of one atmosphere per cm.

3. Evidence is presented to show that in a "steady state" bicarbonate contributes a negligible amount to the diffusion of CO_2.

4. The absorption coefficient for CO_2 in frog skin is 0.73 cc. per cc. and for frog muscle 0.78 cc. per cc.

5. In all of the tissues studied the diffusion of CO_2 is slower than in water. The diffusion coefficients ($K \times 10^4$ in cm.^{2}/minute) at 22°C. for tissues as compared with water are:—water (16°C.), 9.5 (Hüfner, 1897); frog skin, 4.1; connective tissue, 3.7; frog muscle, 6.8; striated muscle (dog), 6.0; smooth muscle (cat), 6.4.

6. The time course of saturation of a tissue with CO_2 is altered in the presence of available base. Non-acidified tissues saturate more slowly than acidified tissues and the rate of saturation is dependent on the CO_2 tension.

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FERTILIZATION AND THE TEMPERATURE COEFFICIENTS OF OXYGEN CONSUMPTION IN EGGS OF ARBACIA PUNCTULATA*

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Since Warburg's (19, 20) pioneer work on *Arbacia pustulosa* extended by Loeb and Wasteneys to *Arbacia punctulata*, it has been accepted that there is a several-fold increase in respiratory rate on fertilization. Recent work on the latter form (16, 17, 21) has set this increase as about fivefold. The early experiments showed further that the respiratory increase could not be equated to the morphogenetic increase, for one could be obtained independently of the other. It was not, consequently, so revolutionary when other eggs were found which showed no respiratory change on fertilization (for details, see Needham (12)), or even manifested a decrease (21). The findings we present are, in a sense, another step in the same direction; for it appears that in *Arbacia* itself the magnitude or even presence of an increase of respiration on fertilization depends on the experimental temperature chosen.[1] The observation that the temperature coefficient of unfertilized egg respiration drops to a value less than half as great on fertilizing (or cytolyzing) has led us, further, to conclusions regarding the catalytic respiration system of these eggs which turn out to be in full harmony with those reached by Runnström (14) from entirely different evidence.

Method

The eggs were prepared and evaluated as described in the preceding paper (7). Each lot was then divided into three portions. One was left untouched; the eggs

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[1] Needham (12, p. 659) mentions work of Fauré-Fremiet hinting at a similar situation in *Sabellaria*.

TABLE I

Sample Protocol

August 7, 1933. Oxygen consumption of *Arbacia* eggs at various temperatures. Each vessel contains 1.0 cc. of egg suspension and 0.2 cc. of N NaOH. Allowed to come to temperature equilibrium before readings were taken—30 min. 54 round trip shakes per min., arc 5.5 cm.

Values in table are corrected manometric pressure differences in mm. Brodie fluid, per hour.

The fertilized eggs did not divide before 110 min. at 14.3°C.

Egg counts: Resting: 256 × 10^3/cc.; fertilized: 192 × 10^3/cc.; cytolyzed: 98 × 10^3/cc. Egg diameter average 73.8 μ.

Time	Fertilized		Resting		Cytolyzed		Temperature	Resting	Fertilized	Temperature
	K = 0.878	K = 0.838	K = 0.953	K = 0.844	K = 0.763	K = 0.841		K = 0.902	K = 0.929	
min.							°C.			°C.
0–20	14.3	14.2	1.8	2.6	1.8	1.0		12.0	30.2	
21–40	14.5	15.0	3.9	2.7	0.7	1.1		11.8	30.3	
41–60	14.0	14.1	3.0	3.1	1.0	0.9		11.8	30.5	
(Q_{O_2})....	65.4	62.8	8.6	9.2	9.2	8.6	14.3	41.9	146	24.0
61–90	13.4	13.3	2.6	2.2	1.8	1.2		10.2	30.2	
91–120	14.2	13.6	2.4	2.5	1.2	1.2		10.1	29.9	
(Q_{O_2})....	63.1	58.2	9.3	7.9	11.7	10.3	14.3	35.6	139	24.0
0–20	27.9	27.2	10.6	11.0	3.8	3.7		10.9	29.9	
21–40	28.1	27.5	11.1	10.9	3.7	3.7		10.9	30.0	
41–60	28.0	27.5	11.0	11.3	3.5	3.5		11.0	29.8	
(Q_{O_2})....	128	120	40.6	36.6	28.0	30.9	21.1	38.9	135	24.0
0–20	45.8	45.1	30.2	28.9	6.0	6.1		11.0	30.2	
21–40	44.8	44.8	29.8	30.0	6.0	5.9		10.8	29.9	
41–60	45.9	44.6	28.6	29.5	5.9	5.8		10.7	29.6	
(Q_{O_2})....	196	185	132	115	47.3	48.2	27.6	38.4	136	24.0

in a second were fertilized by adding freshly shed sperm (the excess was washed off after 15 minutes); and those in the third were cytolyzed by adding three volumes of distilled water. Cytolysis was complete within half an hour. The oxygen consumption of each portion was then determined manometrically. Shaking was at the rate of 54 per minute, arc 5.5 cm., which controls proved adequate to insure oxygen equilibrium without injury (tested by fertilization after 5 hours of shaking). Readings were made every 20 minutes, care being taken that the manometers were in temperature equilibrium before starting.

Aliquots of each portion were subjected simultaneously to two temperatures; and one set was subsequently (after 2 hours, usually) run at one, often two, more temperatures. Tests showed that the order of exposure to high or low temperatures was immaterial, and in practice the order was mixed. The actual values were generally chosen over a wide range, favoring an accurate determination of the Q_{10} ratio. The temperature of the water bath could be changed rapidly by the addition of steam or of ice. The desired temperature was maintained constant within 0.1°C. during a run.

TABLE II

Summary of Data on Oxygen Consumption of Arbacia Eggs at Temperatures between 11°C. and 29.9°C.

Date	Lower temperature	Q_{O_2}			Higher temperature	Q_{O_2}			Q_{10}		
		Resting	Fertilized	Cytolyzed		Resting	Fertilized	Cytolyzed	Resting	Fertilized	Cytolyzed
July 29	16.4	14.0	80.0	9.2	18.2	18.0	82.6	12.0	4.2	1.6	1.7
" 29					21.1	22.3	84.5	10.9	4.3	1.7	1.5
" 25	21.7	12.5			30.6	50.3			4.2		
" 27	17.4	12.6	96.2	10.4	24.2	28.1	133	18.0	3.9	1.9	1.8
August 1	21.0	13.2	65.0	8.9	28.1	41.5	110	17.1	4.3	2.0	2.1
" 2	26.0	24.0	74.4	14.8							
" 3	13.0	8.8	77.8	12.7	24.1	35.3	149	25.5	4.0	1.9	2.0
" 4	15.5	12.0	79.9	8.0	24.0	29.3	135	17.3	3.7	1.7	2.1
" 5	15.0	11.7	70.8	8.1	24.0	38.5	140	15.5	3.8	1.8	1.9
" 7	14.3	8.6	65.4	8.6	24.0	41.9	146		4.2	1.8	
" 7	21.1	40.2	125	29.1	27.6	120	190	48	4.1	2.0	1.9
" 8	13.0	9.1	83.5	11.3	24.0	42.2	135	21.9	4.2	1.7	1.9
" 8	19.9	39.6	123	18.4	29.9	139	222	37.1	3.9	1.8	2.0
" 9	14.6	14.2	101		24.3	55.7	199		4.0	2.0	
" 9					28.5	115	235		4.1	1.9	
" 10	11.0	7.0	68.1		20.3	29.2	139		4.1	2.0	
" 11	21.8	18.9	94.2								
" 12	29.0	53.6	111								
Average...									4.1	1.8	1.9

RESULTS

The data of a typical experiment are presented in Table I. It is immediately evident that at different temperatures the respiration of fertilized, resting, and cytolyzed eggs are in different ratios to one another.

Table II summarizes all the data obtained. A day-by-day tabulation is necessary, since the absolute respiration varies greatly from

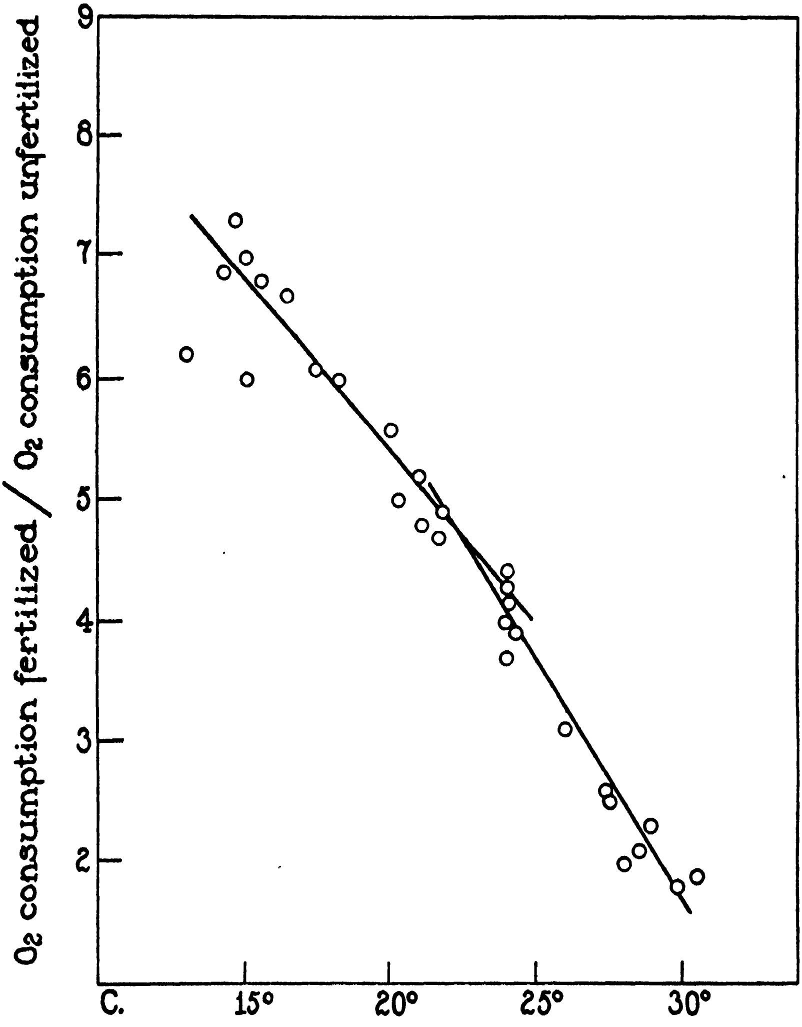

Fig. 1

batch to batch of eggs. It is apparent that the temperature coefficient of the resting eggs (average 4.1) is more than double that of the fertilized (1.8) or cytolyzed (1.9) eggs. The ratio of the rate of oxygen consumption of fertilized to resting eggs is consequently variable. This ratio is plotted against temperature in Fig. 1 and is seen to vary from 8 at 11°C. to 2 at 30°, or, by extrapolation, 1 at 32°. Although a single straight line can be fitted to the observed points, two straight lines intersecting at about 21°C., as plotted, afford much better agreement. That such a break is real, is further supported by its appearance at the same temperature in the plot of μ for fertilized eggs (see Fig. 2).

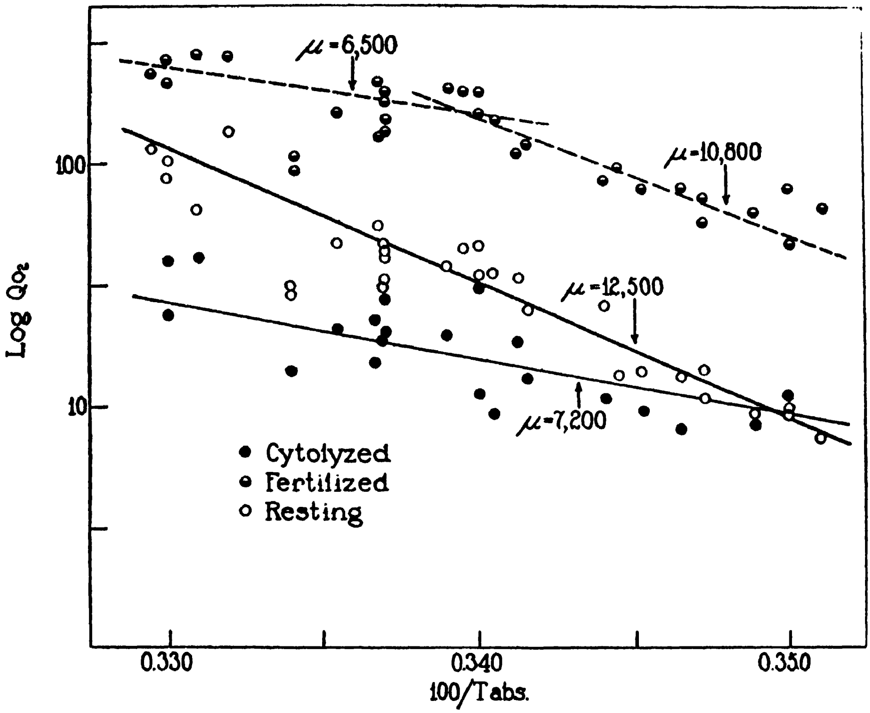

FIG. 2

The critical thermal increment is, perhaps, a more rational expression of temperature relations than the simple Q_{10} (see Crozier (3, 4); and Navez (11)). It is obtained from the van't Hoff-Arrhenius equation

$$V_2 = V_1 e^{-\frac{\mu}{R}\left(\frac{1}{T_2}-\frac{1}{T_1}\right)}$$

where V is the velocity at temperature T, e and R have the usual significance, and μ is the critical thermal increment. The equation is solved graphically, as in Fig. 2, by plotting the logarithm of velocity against the reciprocal of absolute temperature; the slope of the resultant curve is then $-\frac{\mu}{R}$. The numerical value of μ, according to the original theory used in deriving the equation, is a measure of the energy (in calories per mole) required to raise a reactant to its critical level for activity. It has been urged, however, (5) that this reactant may be the catalyst of a particular reaction, or at least a particular catalyst-substrate system (2), which may then be identified by the value of μ. For a complicated reaction chain, as in cell oxidations, the μ value might be that of the slowest (master) reaction. The values found in these experiments are familiar ones. Those of fertilized eggs, 6,500 and 10,800, have, for example, been found for H^+ catalyses; that of resting eggs, 12,500, for iron catalyses (3). The value for cytolyzed eggs is not significantly different from the 6,500 of fertilized eggs.

DISCUSSION

When *Arbacia* eggs are fertilized the respiration may be increased eight times, at 11°, or doubled, at 29.9°. Extrapolation indicates that no increase would occur at 32°C. Since other eggs are now known which do not alter, or actually decrease, their respiration when fertilized, the interesting question presents itself whether similar temperature relations hold for them. It is not impossible that, for each egg, particular temperatures may be found at which fertilization increases, decreases, or does not alter the resting respiration rate. At least it is clear that temperature as well as species must be considered in any future generalizations concerning fertilization and respiration; and further exploration of temperature coefficients in other species and conditions is to be awaited.

It remains to consider the significance of the sharp change in temperature coefficient on fertilizing or cytolyzing the resting egg. This change seems of deeper significance than any alteration of the respiratory rate, since this latter is variable and is partly a consequence of the coefficient change. Also a change in μ indicates a shift in the catalytic system which would not be necessitated by a mere changing of the rate. This shift, moreover, involves the original egg system since great changes occur in 2 to 3 minutes after fertilization, when the sperm is still external to the egg membrane (15). Also, according to Loeb (9), parthenogenetic activation leads to typical changes of fertilization; and our own findings show that cytolysis leads to the same change of coefficient as does fertilization. A particular contribution of enzyme (or substrate) by the sperm is thus, apparently, excluded, so the significant change is to be sought in the egg proper.

It was early pointed out (1, 18) that in a concatenated reaction chain the speed of the total reaction is controlled by that of the slowest member; and, further, as temperature, concentration of H^+ or other substances, etc., are changed, one or another of the individual reactions may become the slowest. Such an interpretation has been successfully applied to many *in vitro* reactions (13). In the case of the egg, the same total reaction—foodstuff plus oxygen forming carbon dioxide, etc.—continues after fertilization, sometimes at the same rate. But whether the rate be increased on fertilization or decreased on cytolysis or unchanged, the factor limiting this rate is different from that operating in the unfertilized egg. A chemical change is further documented by the amazing morphogenetic changes released by activation.

Further analysis of the reactions involved remains hypothetical pending more experimentation. It is not without interest, however, to consider in this connection some of the facts regarding activation. There is evidence that activation depends in part on surface changes. Agents, like narcotics, which can displace substances from adsorbing surfaces, act as activators. Heat, also effective, may partly disorganize surfaces and adsorbed material; and moderate surface injury, produced by cytolyzing agents (NaOH, hypertonic NaCl), likewise activates. The activator, then, tends to disorganize existing cell surfaces, micellar or membrane, and so liberate for free reaction in solution the partly bound and inactive enzyme or substrate. Complete cytolysis would clearly act in the same way, though complicated by actual destruction of some catalytic material. It will be important to determine, for example, if the respiration of eggs activated with hypertonic saline shows the temperature coefficient of fertilized eggs.

Although non-penetrating acids decrease respiration (20), appropriate treatment with penetrating acids leads to activation. This has been studied especially by Lillie (8) for *Asterias*, and he has been led to the view that increased intracellular acidity (possibly just within the membrane) leads to activation. Possibly surface disorganization, with effective increase of reactants, and increased H^+ concentration act together in initiating the new events. It may be noted that the μ values of the fertilized egg respiration are numerically those attributed to H^+ catalyses (3).

Runnström (14) found that carbon monoxide (and cyanide in low concentration), while barely diminishing the respiration of unfertilized eggs, greatly decreased that of fertilized ones. Urethane actually increased resting respiration while cutting that of fertilized eggs to about the same level. He concluded that a limited contact of respiratory enzyme and substrate determined reaction velocity in the inactive egg, hence that considerable enzyme might be poisoned with no decrease in oxidations. With these substances freed to react by colloidal changes in the active egg, the inhibitors manifest their usual effect by binding enzyme. In exactly the same way, the high temperature coefficient of resting eggs might document the influence of temperature on the state of adsorption, while the lower one, of structurally disorganized eggs, measures the direct effect of temperature on the speed of reaction. Change in colloidal state, *e.g.* gelation of gelatin, or membrane structure, *e.g.* in nerve (6), often shows a remarkably high temperature coefficient; and the fraction of free reactants released from the adsorbed bulk would be similarly sensitive to temperature. Further temperature studies should help to check these interpretations.

We wish to thank Dr. Ralph S. Lillie for his kind assistance.

SUMMARY

The eggs of *A. punctulata* have a high temperature coefficient in the resting state: $Q_{10} = 4.1$.

On fertilization and on cytolysis the temperature coefficient falls to less than half the resting value: $Q_{10} = 1.8$ and 1.9 respectively.

The factor by which oxygen consumption increases on fertilization is a variable, its magnitude depending on temperature as well as on egg species. It is nearly ten times greater at 11°C. and only double at 29.9°C. By extrapolating to 32°C. there would be no increase on fertilization.

Critical thermal increments common to many oxidations, 6,500, 10,800, and 12,500, have been found.

The possible significance of these results is discussed in relation to the catalytic mechanisms and structural organization of the egg cell.

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THE GROWTH OF CUCUMIS MELO SEEDLINGS AT DIFFERENT TEMPERATURES

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I

The work of Julius Sachs (10) published in 1860, and in particular his discussion of the results, served to introduce two concepts into plant physiology, that of minimal, optimal, and maximal temperatures, and that of the grand period of seedling growth. Each of these phenomena has received the attention of numerous workers since that time but there have been relatively few attempts to combine the two fields of interest and to follow the course of growth of seedlings cultured in darkness at several constant temperatures throughout their entire growth period. The work of Sierp (11), Hamada (4), and Silberschmidt (12) on the growth of oat coleoptiles belongs in this class, as does that of Edwards, Pearl, and Gould (1) on *Celosia cristata* seedlings. Silberschmidt also tested pea and rice seedlings and contributed an interesting analysis of the problem.

The present paper deals with the growth of *Cucumis melo* seedlings in darkness at seven constant temperatures between 15° and 40°C. inclusive. While we have accumulated in this laboratory during the past 10 years a considerable volume of unpublished observations on the effect of temperature on the growth of canteloup seedlings, so far as is known the only published work on the temperature relations of growth in this species are those of de Vries (14) who found greater growth at the end of a 48 hour period at 37.2° than at any of the other three temperatures he tested. Gregory (3) has made interesting temperature tests of another sort on *Cucumis sativus* seedlings grown in light which indicate a much lower optimal temperature range than was found in de Vries' experiments or in those presented here.

The cultural methods used in the experiments reported here are essentially those used in other experimentation on canteloup seedlings reported from this laboratory (2, 6, 8) and they may be stated briefly as follows: Seeds from a single melon were freed from their testas, weighed, and only those whose weights fell between 0.0200 and 0.0240 gm. were used. They were immersed 1 minute in 1:1000 $HgCl_2$ solution, rinsed once, and soaked 3 hours in sterile distilled water in individual vials. They were planted in previously sterilized glass tubes, 44 cm. long and 2 cm. in diameter, containing 25 cc. of 1 per cent agar (the 30° tubes contained 40 cc.) made up in Knop's mineral salt nutrient solution, instead of distilled water as in the other experiments. Another difference in the procedure used here consisted of forcing 80–90 cc. of air into each tube daily, but comparative tests (8) showed that this did not improve growth. The tubes were kept in darkness in constant temperature chambers and the lengths of the straight portion of the hypocotyl, exclusive of the curved portion at the top, were measured under non-actinic ruby light regularly at 24 hour intervals from the time of planting of the seeds.

The purpose of the present paper is to record the description of certain experimental observations made in the course of development of the program of investigation of seedling growth and duration of life, which has been in progress in this laboratory since 1925, and to analyze quantitatively the effect of temperature upon the growth of *Cucumis melo* seedlings under a particular set of experimental conditions. A more extensive series of experiments of this kind will be reported later, in which *Cucumis* seedlings were grown with and without mineral salts, at five constant temperatures, and in which the respective durations of life were observed.

II

Table I shows the mean lengths of *Cucumis melo* hypocotyls for various intervals after planting at six constant temperatures. Germination and a little root growth occurred at 40° but the hypocotyls did not become differentiated. There is a little irregularity in the 20° data near the end of the growth cycle because observations on two series of experiments were combined; in one series no readings were made on the 16th day and in the other none were made on the 17th. Although the numbers of seedlings used in these tests are small, nevertheless there was good agreement between the values within each series and those obtained by repetition. Except at 15° two or more series were run at each temperature and the data were combined.

The data show that during the first 2 days of observed elongation of the hypocotyl both the 37.5° and 35° seedlings were taller than those at 30°, but their rapid initial growth was not sustained long, and the 30° cultures finally grew taller than any others. Although the 25° cultures grew more slowly than at 30° nevertheless they finally grew nearly as tall. At 20° about 4 days were required for differentiation and the first appreciable elongation of the hypocotyl. Growth was

TABLE I

Mean Height of Cucumis melo Seedlings at Six Constant Temperatures

Interval after planting	15°	20°	25°	30°	35°	37.5°
days	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
2	—	—	2.6	3.1	8.9	5.5
3	—	—	14.1	24.0	41.4	25.0
4	—	2.3	51.1	77.0	89.1	57.2
5	—	7.8	101.8	121.5	127.4	91.5
6	—	13.7	142.3	166.4	159.2	118.5
7	—	31.4	176.6	205.3	178.2	136.0
8	—	59.0	203.4	225.5	187.5	146.9
9	—	92.3	212.1	236.9	191.5	151.9
10	—	120.6	219.0	240.4	192.8	153.9
11	—	143.8	221.2	242.8	194.2	154.2
12	—	159.5	223.6	243.8	194.8	—
13	1.0	167.9	224.1	—	—	—
14	1.0	172.2	—	—	—	—
15	—	173.1	—	—	—	—
16	2.6	178.7	—	—	—	—
17	3.6	177.5	—	—	—	—
18	5.3	177.8	—	—	—	—
19	7.1	—	—	—	—	—
21	13.0	—	—	—	—	—
23	21.9	—	—	—	—	—
25	35.1	—	—	—	—	—
27	49.0	—	—	—	—	—
29	61.4	—	—	—	—	—
31	69.1	—	—	—	—	—
33	72.3	—	—	—	—	—
35	73.0	—	—	—	—	—
37	73.4	—	—	—	—	—
No. of seedlings...	7	13	17	18	17	11

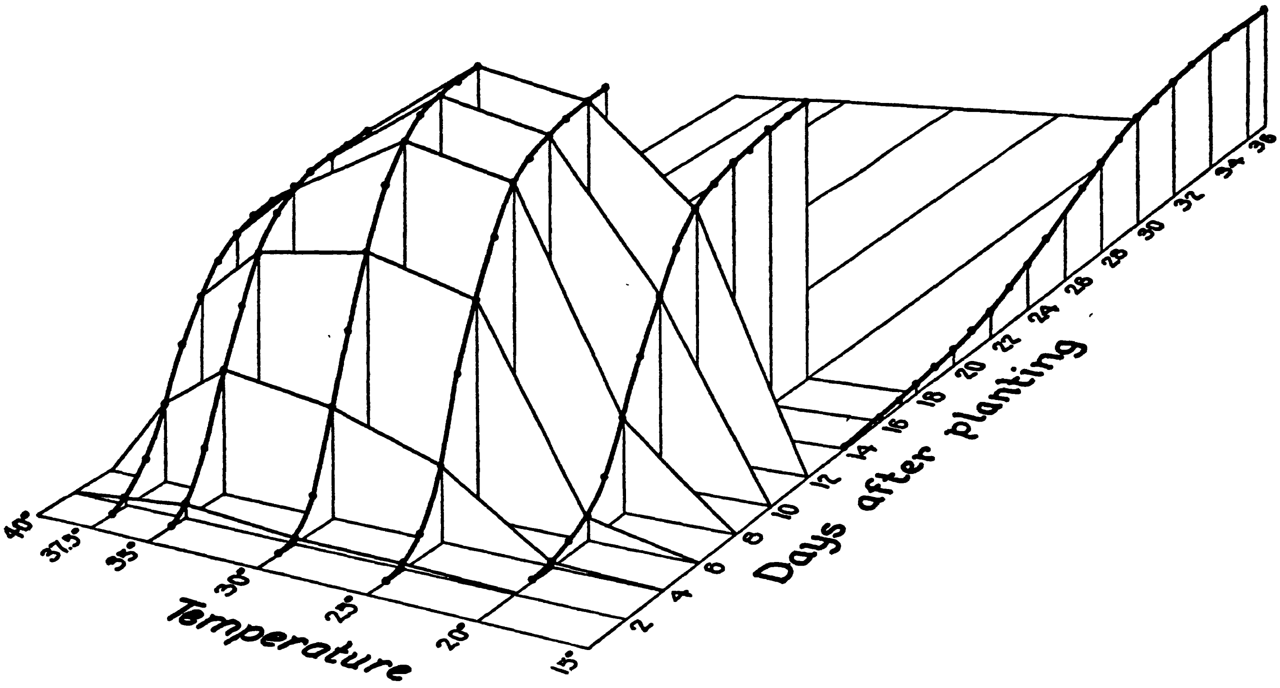

FIG. 1. Three dimensional diagram showing the observed heights of *Cucumis* hypocotyls (represented by heights of plotted points) as observed at six constant temperatures for total growth periods of different durations.

still slower than at 25° and the final height was less but they grew more rapidly and to a greater height than at 15°.

If, for purposes of comparison, one considers these sigmoid growth curves as made up of three portions, a lower part which is convex downwards, a nearly straight portion on either side of the point of inflection, and an upper part which is concave downward some interesting differences in form among the curves appear. The lower portion is most prominent at the lower temperatures, and as one examines the curves for successively higher temperatures it becomes less conspicuous. An examination of the slope of the central portions shows that the 30° seedlings grew most rapidly of all, and the farther removed from 30° either above or below that a tested temperature lay, the lower was the maximal growth rate observed. As for the upper parts of the curves, little difference in form is to be seen. A study of the increments of growth serves to confirm these relations but this does not require further discussion here.

The three dimensional diagram in Fig. 1 has been constructed as though the curves of Table I had been traced on some material like cardboard, then cut out and made to stand perpendicularly and in proper order. Intersecting these planes at right angles is another set of graphs which shows the heights attained by the hypocotyls after growth periods of certain durations at the several constant temperatures. This gives rise to a system of three coordinates; the temperature scale is laid off along one axis, the number of days after the planting of the seeds along another, and the heights of the plotted points above the plane formed by the first two coordinates represent the mean heights of hypocotyl grown at a given temperature for the period of time indicated. The curves passing through these points have been smoothed graphically. An attempt has been made to allow for the effect of perspective in drawing this diagram; a separate scale of height, proportionate to the distance from the vanishing point on the left, has been used for each temperature, and all the lines of the time and temperature coordinates have been made to converge on two vanishing points, one at the left, and one behind the diagram.

Three dimensional diagrams of this sort have been used by Rahn (9) to show the temperature relations of enzyme action and similar phenomena, and have long been a commonplace of statistical literature, particularly in the writings of Karl Pearson and his associates, and of A. J. Lotka.

An inspection of the transverse planes connecting culture periods of the same duration provides a means for comparing graphically the relative heights of the seedlings. The high initial growth rates prevailing at 35° stand out clearly, and the greater relative growth that occurs at 30° as time goes on is shown by the slopes of the lines that connect the two growth curves. Shifts of the optimal temperature with time were first reported by Lehenbauer (5) for maize seedlings, and they occur in the data of Talma (13) for *Lepidium sativum* roots, in the work of Silberschmidt (12) for oat coleoptiles, and in particular in his experiments on pea epicotyls which had their highest initial growth rates at 25° but which attained their greatest length at 12.5°C. The data of Edwards, Pearl, and Gould (1) for *Celosia* show similar relations as do the present data for *Cucumis*.

III

While Fig. 1 permits a rough visual appreciation of the effect of temperature on growth in these experiments, further analysis is essential to an understanding of the matter. It is obvious that there are many variables involved in the growth of any organism. In the present case the records taken permit an analysis of three of the basic variables in all growth phenomena; namely, *yield* (here measured by length of hypocotyl), *time*, duration of whole growth period, defined as the time from planting to cessation of elongation of the hypocotyl and, derivatively from these two, the total *time rate* of growth (*i.e.* the amount of yield per unit of time). Study of the data of Table I brings out the following relationships.

1. As the temperature deviates from the observed optimum[1] (30°),

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[1] Since the literature indicates a considerable confusion of thought and diversity of usage regarding the concept of the optimum temperature for growth, it may be well to state that in this paper we use the term to indicate that temperature at which the greatest total amount of growth activity occurs. Under the conditions of our experiments with *Cucumis melo* it is a matter of indifference as to whether (*a*) mean time rate of growth (yield per unit of time spent in growing) or (*b*) mean absolute yield (length of hypocotyl) at the end of growth, be taken as the index or measure of "total amount of growth activity." This may not necessarily be the case with other species or under other experimental conditions.

in either the plus or minus direction the *yield* is reduced. The minus deviations in yield, measured in units relative to the yield at the optimum, are fairly closely proportional to the relative deviation in temperature (neglecting signs and having regard to errors of sampling) at temperatures near the optimum, but are in excess, proportionally to the temperature deviations, at the outer temperatures remote from the optimum.

2. As the temperature deviates in the minus direction from the optimum the *duration* of the whole growth period tends to be prolonged, again about proportionally (relatively) to the temperature deviations at temperatures near the optimum, but in excess at the temperatures more remote. At temperatures above the optimum (so far as the data go) the duration of the growth period appears to be approximately constant and the same as that at the optimum.

3. The above relationships at once suggest that the total *time rate* of growth (yield per unit of time over the whole growth period) will be found to follow a parabolic relation to temperature, of the general form

$$R = a + bT + cT^2, \tag{i}$$

where R = rate as above defined, and T = temperature in C° above zero.

This suggested relationship turns out to be the fact, when the rates computed from the data of Table I are fitted by least squares with such a parabola. The equation so computed is

$$R = 4.864T - 0.082T^2 - 53.274, \tag{ii}$$

where R = average growth in mm. of hypocotyl *per diem* over whole period of growth, and T = temperature in C°. Putting $\frac{dR}{dT} = 0$ from equation (ii) it appears that the temperature at which the average time rate of growth of *Cucumis melo* is at its maximum under the conditions of these experiments, is 29.74°C.

The observations and fitted curve are shown graphically in Fig. 2. The fit is an obviously reasonable one, considering the magnitude of the data.

In the discussion up to this point we have taken as a single numerical measure of total growth activity of the seedling—a logically necessary statistic if growth activity is to be related mathematically to temperature—the mean total time rate of growth (yield per unit of time) as defined above. This procedure involves two postulates; namely, (1) that the process called growth is a continuous one, at, however, widely varying rates, from the time the dry seed is planted and begins the imbibition of water, until the process ends, with the cessation of enlargement of the plant. In other words this postulate assumes that there is no biological discontinuity between the processes of germination on the one hand and *visible* growth of the plant and its organs on the other hand. (2) That the mean total time rate of growth (as defined earlier and computed in the present instance by dividing the final

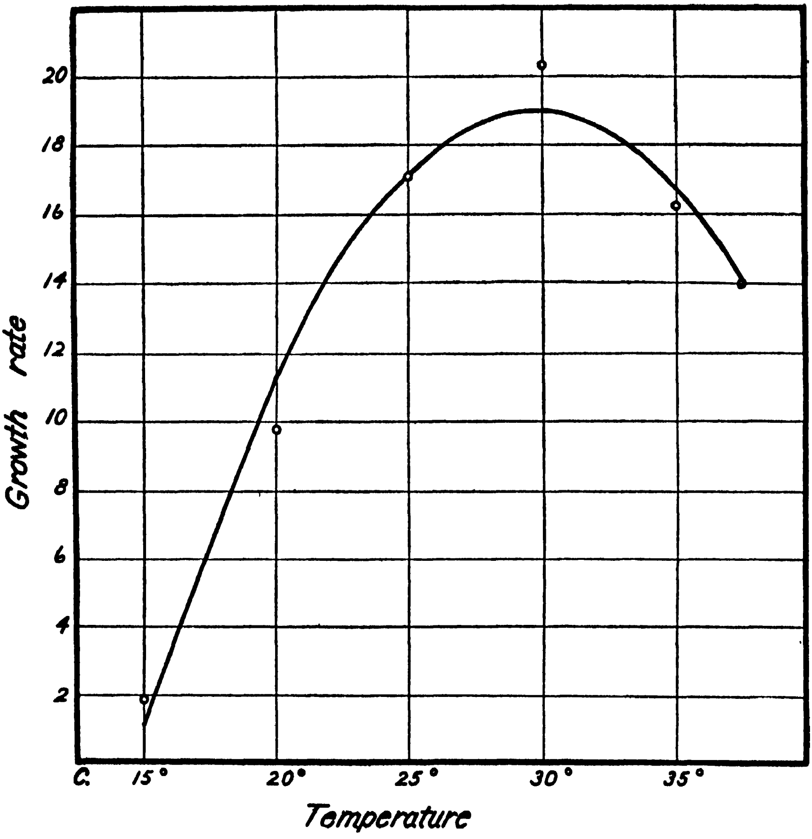

FIG. 2. Change of time rate of growth of *Cucumis melo* with temperature. Circles give the observations, and the smooth curve is the graph of equation (ii).

height of the hypocotyl by the whole number of days of growth from planting to the cessation of growth) is a reasonable and efficient *single* numerical index of total growth activity. While these two postulates seem reasonable, it is evident that they are by no means the only ones possible in the premises. Other measures of growth rate might be taken; germination might conceivably be regarded as something

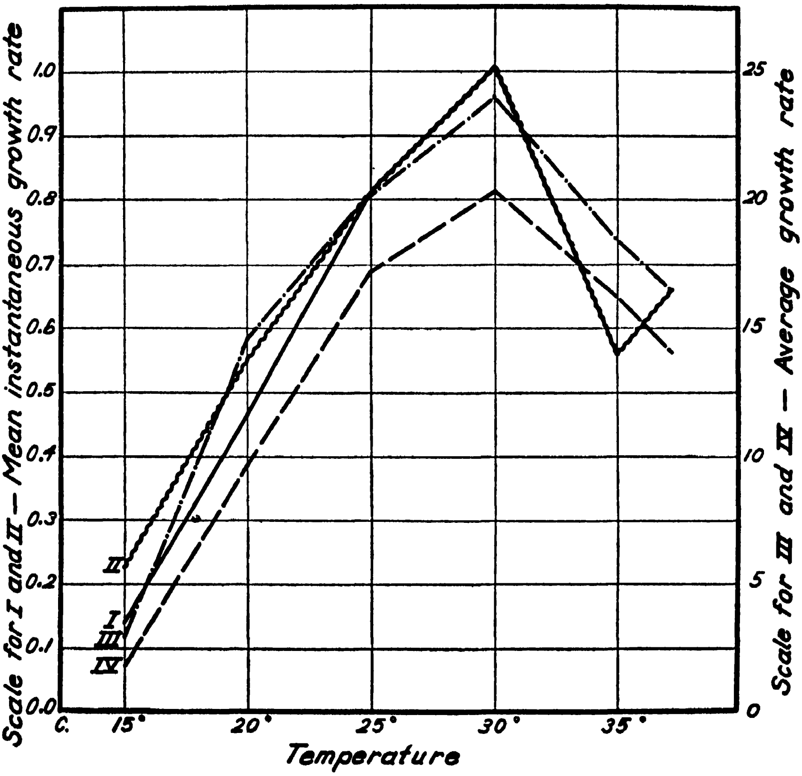

FIG. 3. Showing the similarity of results under different postulates as to growth rates and times.

biologically different from visible growth, disparate, and discontinuous with it. Would the results be different so far as concerns the main point at issue—the relation of growth of the seedling to temperature—if postulates different from those used above were adopted?

To test this question three different procedures were tried, in addition to the one already described above. These were:

I. To compute for each temperature the mean daily relative growth rate for that day by finding the fraction which the absolute mean increment for the day was of the growth already attained at the beginning of the interval. Then their daily mean relative growth rates were averaged for the whole period from planting to the cessation of growth. This procedure corresponds in principle to determining the mean instantaneous growth rate over the whole period, but is done on the basis of the gross finite differences of the observations, rather than upon differentials.

II. The same procedure as in I, except that only the period of observed, measured elongation of the hypocotyl is used, instead of the whole period from planting to the cessation of growth as in I.

III. The same procedure as was used in computing the data of Fig. 2, except that the rates were calculated on the basis of only the period of observed, measured elongation of the hypocotyl.

The results of all four methods of dealing with the data (the original method and I, II, and III) are shown graphically in Fig. 3, the scales of plotting being so adjusted as to bring all the lines near together, since the interest in the case pertains to relative rather than absolute values.

It is evident from Fig. 3 that such differences in theories of the growth process, and views of an appropriate measure of the growth rate as are embodied in it make no essential or important difference in the net result regarding the relation to temperature of growth in *Cucumis melo* seedlings under the conditions of these experiments.

IV

The data of Table I and Fig. 1 indicate that temperature affects growth differently in different parts of the whole growth period or cycle. The nature of these changes is clearly shown in Table II, in which two sets of percentages are presented. The first of these gives the approximate percentage of the final mean total yield at each temperature which the plants in that temperature series have achieved at the end of the first quarter, the first half, and the first three-quarters of their own total growth periods. If the growth proceeded uniformly with time, and was the same relatively in all temperature series, the figures in the left half of Table II would be all 25 on the first line, 50 on the second line, and 75 on the third. The second set of figures, in the right half of Table II, gives the approximate percentages which the achieved yield in each temperature series was of the achieved yield in the 30° series (the optimum temperature in respect of total yield) in one-quarter, one-half, and three-quarters of the total growth period of each series. The figures show, for example, how the yield of the 20° series compared with that of the 30°, when each had accomplished one-fourth of its total growth period. The percentages are approximate in Table II because, in interpolating times and yields, we have assumed that growth proceeded at a constant rate between any two recorded observations. This is not strictly true, especially in some parts of the cycle, but the error implicit in this assumption is negligible for present purposes. In all of the computations the total length of the growth period is taken as from planting to the cessation of growth. That is to say, germination is counted, in respect of time, as a part of growth.

TABLE II

Approximate Relative Yields (Mean Hypocotyl Lengths) at Stated Relative Times, and in Proportion to 30° Yields at the Same Relative Times

Percentages of total growth period	Percentages of own total yield						Percentages of own yield to 30° yield at same relative times					
	15°	20°	25°	30°	35°	37.5°	15°	20°	25°	30°	35°	37.5°
25	0	2.8	10.4	9.8	21.3	13.1	0	21.0	97.3	100	172.5	83.9
50	8.4	51.9	71.2	68.3	81.7	68.1	3.7	55.5	95.8	100	95.7	63.1
75	73.1	95.6	97.0	97.2	98.3	96.1	22.6	71.8	91.7	100	80.8	62.5
100	100	100	100	100	100	100	30.1	72.9	91.9	100	79.9	63.2

From Table II the following points may be noted.

1. At no temperature did the seedlings in these experiments attain 25 per cent of their final total yields in the first quarter of their growth period. They came nearest to it in the highest temperatures, above the optimal.

2. When a half of the total growing period has been completed the seedlings in all the series except the 15° have achieved more than 50 per cent of their final total yields. The same thing is true, *mutatis mutandis*, for the first three quarters of the total growth period, except

that there the 15° series with 73.1 per cent of its total yield achieved is not significantly below the even 75 per cent.

3. In general it is plain that in these experiments at all temperatures above 20° the greatest growth activity was concentrated in the second quarter of the total growth period. At 20° the same thing was true, taking the figures literally at their face value, but the difference between the growth activities in the second and the third quarters at this temperature was so small as to be practically insignificant. At 15° the greatest growing activity was in the third quarter of the cycle. Also at this temperature the activity was much greater in the fourth quarter than at any other of the observed temperatures. In short, as the temperature increased in these experiments the time of greatest growing activity tended to be pushed farther and farther back towards the beginning of the cycle.

4. The same rule is manifested in another way in the second half of Table II. At the end of the first quarter of their respective growth cycles the 35° seedlings, for example, had an average yield nearly twice as great as the 30° seedlings, though in the end their yield was only 79.9 per cent of the 30° lot.

5. In these experiments temperatures far below the optimum (15° and 20°) affected growth activity more adversely in the first quarter of the cycle than in any other period of the cycle. Temperatures above the optimum (35° and 37.5°) affected growth activity more adversely in the third and fourth quarters of the cycle than in any other part of the cycle.

6. At all temperatures tested and in all parts of the growth cycle (save only the first quarter at 35°) growth as measured by yield was less than in the corresponding part of the cycle at 30° in these experiments.

7. It is possible that the short duration of the relatively high growth rates observed at 37.5° and 35° may be due to a rapid oxidation of the available food materials by the high rates of respiration which one may presume these temperatures induce, and which renders the food supply inadequate for further growth fairly early in the growth cycle. The present data, however, furnish no material by which this possibility may be crucially tested, and it is therefore idle to discuss it further.

V

The results of this study suggest that anything approaching a thorough understanding of the phenomenon of growth is not likely to be reached until we have comprehensive and exact observations available regarding the cellular activities going on during the process. In so comparatively simple a biological structure as a seedling (simple, that is to say as compared with a human fetus) growth is far from a homogeneous phenomenon. Not all parts of the growing organism are growing at the same time, or at the same rate. While much progress has been made in recent years in getting an understanding of the gross quantitative relations of growth, we still know next to nothing of the real underlying biology of the process, the cellular activities involved, etc.

Summarizing the results it appears that seedlings of *Cucumis melo*, grown under carefully controlled experimental conditions at seven constant temperatures, show the optimal temperature to be approximately 30°C. (29.74° from the graduated data). The mean time rate of growth (millimeter increase in length of hypocotyl *per diem* over the whole growth period) is a parabolic function of temperature. As the temperature increases, within the limits of these experiments, the period of maximum growth activity tends to fall earlier in the whole growth cycle.

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THE INFLUENCE OF ENVIRONMENTAL TEMPERATURE ON THE UTILIZATION OF FOOD ENERGY IN BABY CHICKS*

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INTRODUCTION

The influence of the environmental temperature on the vital functions of poikilothermic organisms has been extensively investigated. Van't Hoff's rule for the relation of temperature and velocity of chemical action has been applied to physiological problems, for example, the development of sea urchin eggs by Abegg and to photosynthesis by Matthaei (Verworn, 1922). Crozier (1924–25) and coworkers have demonstrated that Arrhenius' equation for the velocity of irreversible chemical processes as functions of temperature expresses very well the influence of environmental temperature on a great number and variety of vital processes in organisms or parts of organisms. Stier (1932–33) has shown that one may consider the inheritance of distinct temperature characteristics for the frequency of respiratory movements even in mammals at least during a time when the temperature regulation is not yet developed as in new born mice.

Temperature optima have been observed for the development of various poikilotherm organisms, as for *B. tuberculosis* 37–38°C. by Koch. Surface yeast according to J. Albauer grows fastest at 33–34°C.; for sediment yeast Pederson found maximum growth rate at 29°C. (Mayer, 1927).

The main object of the investigation reported in this paper was to study how the environmental temperature affects growth rate, food consumption, and the conversion of food to body substance in homoiotherms where the environmental temperature has little if any direct effect on the temperature of the majority of body cells.

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* The experimental work for this study has been carried out in the Division of Animal Husbandry, in coöperation with the Division of Poultry Husbandry at the College of Agriculture of the University of California at Davis. It has been made possible by the financial contribution of the California Committee on the Relation of Electricity to Agriculture.

The influence of the environmental temperature on the metabolism of homoiotherms has been studied mainly on fasting animals to eliminate influences of food intake on the result of the experiment.

These investigations point to the existence of a certain critical environmental temperature below which a cooling of the environment causes an increase in metabolism and above which the metabolism is practically independent of changes in the environmental temperature.

The broken line, *abc*, on a temperature-metabolism chart (see Fig. 1) illustrating this behavior of homoiotherms may be interpreted as a result of two curves which in the first approximation are straight lines, one (inclined) representing the heat requirement for maintaining the animal's body temperature at a constant level, and the other (horizontal) indicating the minimum heat production of the animal. The critical temperature Tc_1 is then defined as the temperature at which the two lines coincide. If the animal is fed, the level of the minimal heat production is raised for the amount of the heat increment, or the specific dynamic action of that food, C_m and C_p. This increase in the minimal heat production lowers the critical temperature to Tc_2 for maintenance and to Tc_3 for full feeding.

The influence of variations in temperature on the animal receiving a certain food was studied on dogs by Rubner (1902), who condensed the results of this investigation to his so called compensation theory. According to this theory, the metabolism below the critical temperature for the full fed animal Tc_3 is not affected by food intake (no specific dynamic action of the food). Above the critical temperature for fasting, Tc_1, the specific dynamic action of the food is constant; *i.e.*, independent of the changes in the environmental temperature. In the region between these two critical temperatures, the specific dynamic action increases in proportion to the increase in temperature. Rubner's compensation theory seems not to have been given much attention in experiments. In general, the investigators were careful only to keep their animals above the critical temperature in order to obtain results independent of temperature changes.

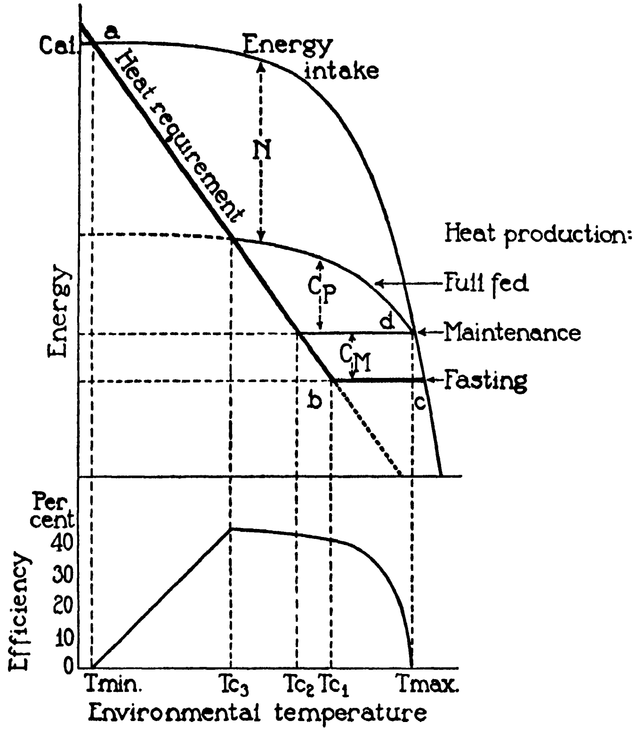

FIG. 1. Scheme of energy transformations in homoiotherms as a function of environmental temperature. The inclined straight line marks the heat requirement; *i.e.*, the amount of heat which would be necessary to maintain the animal's body temperature if the animal were an ordinary thermostat. Since the radiation depends on the difference of the 4th powers of temperatures this line is actually a curve. For small differences in temperature, however, it does not deviate considerably from a straight line.

Below the critical temperature, Tc_1, the heat production of the fasting animals follows the line of the heat requirement; above that temperature it is independent of further changes in temperature (minimum heat production). At high environmental temperatures the heat production rises again with rising temperature. This effect is omitted in the scheme.

C_m indicates the specific dynamic action of the food for maintenance which brings the heat production to a higher level and lowers the critical temperature to Tc_2.

C_p illustrates the specific dynamic action of the production food calculated to be 33 per cent of the energy intake above maintenance.

The difference between the energy taken in and the heat produced is the net energy N.

The net energy in per cent of the total food energy is the efficiency. It is maximum at Tc_3; *i.e.*, at the critical temperature for the full fed animals.

Our study was undertaken to investigate the influence of temperature changes on the energy transformation of fed animals including the range below the critical temperature which has commonly been avoided. Instead of giving definite rations, as has been done in most former experiments, we let the animal itself determine the amount of food intake. In this way, we studied the appetite in relation to the environmental temperature, growth rates, and energy utilization, as an important variable which determines in a great many cases the actual level of energy transformation in animals.

From our own experience, we know that our appetite is greater in cold than in hot weather.[1] Thus, if enough food is available, the increased requirement for temperature regulation at lower environmental temperatures is paralleled by an increased energy intake. This parallelism is, however, not complete. The lower the environmental temperature, the higher is the heat requirement for temperature regulation. The energy intake to the contrary cannot increase indefinitely because the capacity of animals for eating, digesting, and absorbing food is limited. It is, therefore, to be expected that at sufficiently low environmental temperatures the energy intake of the animal can no longer keep pace with its heat requirement and the animal starves to death even though it eats to capacity. The temperature at which the curve of the heat requirement and the curve of intake of metabolizable energy coincide (point *a* in Fig. 1) is the minimum temperature ($T_{min.}$) under which the animal is able to maintain its life continuously. At this temperature, naturally, no energy will be available for production of body substance. Thus the net energy[2] and consequently the total efficiency of the animal as a converter of food energy will be zero. The production of body substance will also be zero at extremely high environmental temperatures when the appetite is decreased to such an extent that the energy intake covers only the minimal heat production for maintenance (*d* in Fig. 1). This temperature is the maximal temperature ($T_{max.}$) which an animal can survive continuously.

Between the two extreme temperatures at which no production can take place because too much energy is lost at one and too little is taken in at the other, there should be a temperature at which the production is maximum, and one at which the total efficiency; *i.e.*, the quotient of net energy to total energy intake, is maximum. In our schematic graph, Fig. 1, they are both at Tc_3 the critical temperature for maximal food intake.

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[1] The contrary is true for grasshoppers which, in an experiment by Parker (1930), consumed at 37°C., 2.5 times as much food as at 27°C.

[2] Net energy = heat of combustion of produced body substance.

Method

From a hatch of White Leghorn chicks 5 days of age, five individuals were selected and banded as the experimental group, and eight as the control group. Sex did not appreciably affect the results since the number of male and female chicks in each of our trials happened to be almost equal as shown by investigation several weeks after the trial when the sex characteristics began to develop. Also in these young birds the differences of sex with relation to growth seem not to be considerable according to Jull and Titus (1928) who found that for the first 6 or 8 weeks the female chicks weighed practically the same as the males. The controls were kept in a brooder which was so constructed that the chicks could stay at a temperature of 35°C. or go into an outer compartment at room temperature as they chose. This choice was somewhat influenced by the fact that the food was kept in the outer compartment. The experimental chicks were kept in the respiration chamber at constant temperature and humidity, except for about 15 minutes in the morning and evening, which time was required for obtaining the individual weights, changing the food and water, and collecting the excrement.

Each experiment for one level of temperature lasted 9 days and was followed by the determination of the fasting metabolism. The experiments at the various temperatures were carried out in the following sequence: 38°, 27°, 40°, 32°, 21°, 40°C.

The food was considered qualitatively adequate for every requirement.[3] Portions of 150 gm. were weighed and stored in glass jars before the trial started. The food was given every morning in a food box which was constructed to prevent the birds from contaminating the food by excrement. The amount of food given was abundant, intake being limited only by the appetite of the chicks. The remaining food was taken out in the evening and put into a drying oven at 105°C. for the moisture determination so that the amount of the dry matter eaten by the group was known for each day. No food was given during the night.

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[3] The food was prepared in pellet form according to the following recipe:

Mix 25 parts of ground whole wheat,
25 parts of ground whole yellow corn,
25 parts of ground whole barley,
15 parts of fish scrap with 65 per cent protein,
5 parts of dry skim milk,
5 parts of ground bone.
To 100 parts of this mixture add:
2 parts of pulverized lime stone (96 per cent $CaCO_3$),
½ part of salt (NaCl),
1 part of cod liver oil.

The excreta were removed every day from the pan below a false floor of chicken wire, dried at 105°C., and weighed before cold. The dried excreta for the whole period of 10 days were stored in a glass jar and a composite sample was analyzed.

Food and excreta were analyzed for N according to Kjeldahl and for C by the method of wet combustion with potassium dichromate. The heat of combustion was determined in an Emerson fuel calorimeter.

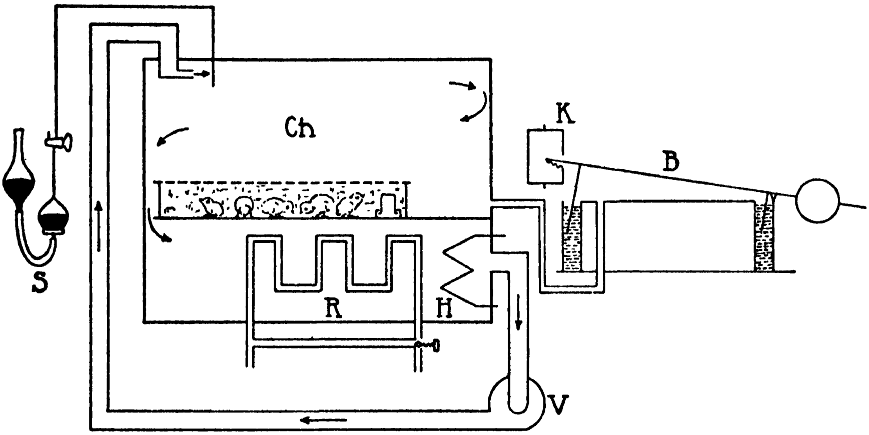

FIG. 2. Climatic cabinet. *ch*, chamber; *R*, refrigerator coil; *H*, electric heater, *V*, air circulation; *B*, pressure regulator; *K*, kymograph drum; *S*, sample for gas analysis.

The respiratory exchange of the experimental chicks was determined in a closed chamber constructed as a climatic cabinet (Fig. 2). This cabinet is a horizontal metal cylinder 91 cm. inside diameter and 122 cm. inside length covered with cork for insulation. Five electrical heaters (*H*), each controlled by a separate outside switch, are installed in the chamber, giving a total heating power of 2250 watts. One of the 500 watt units is a control element operated by an automatic temperature regulator. A system of brine coils (*R*) for the regulation of the humidity is mounted in the lower part of the chamber. The flow of brine through these coils is under the control of a wet bulb air thermometer inside the chamber which influences a diaphragm valve, opening and closing a by-pass in the brine duct. A fan (*V*) circulates the air inside the chamber over the cooling and heating systems. A heavy round door with a thick walled glass window is mounted on a hinge at the front of the chamber and may be screwed against a rubber gasket for an air-tight closure.

The air-conditioning devices produce regulatory oscillations of the temperature and humidity inside the chamber, which would cause resultant fluctuations in air pressure if the volume remained constant. If there is the slightest leakage these variations in pressure tend to cause an exchange between the air in the chamber and the air outside. These changes in pressure are practically avoided by a regulator (*B*) providing a calibrated buffer volume.

A sample of the air in the chamber is taken at the start and at the end of a period. This gas sample is analyzed for CO_2 and O_2 in a modified Haldane apparatus (Kleiber, 1933 *b*).

The volume of the chamber has been determined by introducing volumetrically measured amounts of CO_2 from a bomb and determining the difference in CO_2 concentration thus effected; it has also been measured by burning known amounts of ethyl alcohol in the chamber and determining the increase in CO_2 concentration due to this combustion. In five tests by introduction of CO_2 gas the volume was found to be *890 ± 13.5 liters.* In seven tests with combustion of alcohol, the determined volume was *898 ± 11.9 liters.*

RESULTS

(*a*) *Influence of Temperature on the Growth Rate of Baby Chicks*

The chicks were weighed individually each morning after having been 12 hours without food, and every evening. The average morning weight for all experimental groups at the start of the experiment was 55 gm. with extremes from 53 to 58 gm. At the end of the experiment, the body weight of the 15 days old chicks differed considerably according to the temperature at which they had been kept, ranging from 79 gm. each for the group kept at 40°C. to 99 gm. each for the group kept at 21°C.

In Fig. 3 the average growth rate for 6 to 15 days of age is plotted against the environmental temperature. The growth rate of all controls for which, of course, the abscissa has no meaning, is averaged and drawn as a dotted line.[4] From the value for the growth rate of the experimental chicks the solid curve is interpolated. The curve shows a tendency to reach a maximum which, however, would be at a temperature lower than 21°C.

The curve of the growth rate of the experimental chicks reaches the average level of the growth rate of the controls at a temperature between 32°C. and 38°C.

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[4] The growth rate of the controls simulates a relation to the temperature at which the experimental chicks were kept. The dot and dash line in Fig. 3 is the result of interpolation by the method of least squares. This line used as a basis of comparison leads, however, to the same conclusions as the line which expresses the average of the controls.

At an environmental temperature above 32°C., the experimental chicks had a lower rate of growth than the corresponding controls. Below this temperature the growth was the more stimulated the lower the environmental temperature.

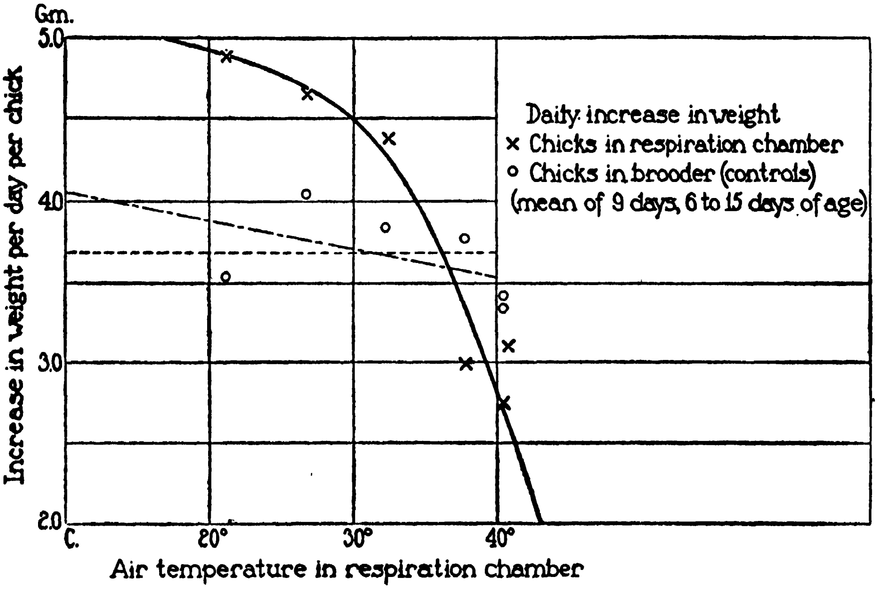

FIG. 3. Growth rate and air temperature.

Since the daily increase in weight in growing animals depends on the weight itself, the relative rate of growth is more satisfactory than the absolute rate for comparing the velocity of growth in animals of different size.

The relative rate of growth for our chicks has been calculated according to the definition given by Brody (1926–27, p. 641) for the accelerating phase of growth:

$$\frac{dW}{dt} = k.\,W$$

The meaning of the terms in our calculation was:

W = body weight in grams.
t = time in days.
k = relative rate of growth.

The integrated form of the function

$$k = \frac{\ln W_2 - \ln W_1}{t_2 - t_1} = 2.30259 \frac{\log W_2 - \log W_1}{t_2 - t_1}$$

has been used, basing the calculation on the weight at the age of 5 days, the average of the weights at 10 and 11 days, and the weight at 15 days of age.

Table I shows the results of this calculation. The relative growth rate is greater the lower the environmental temperature. In all but one case (38°C.) the relative growth rate is higher in the second period (10.5 to 15 days of age), than in the first one. This behavior is also found in the controls except that the controls for one trial out of 6 had a slower relative rate of growth in the second period. All other chicks showed an acceleration of growth with increasing age greater than that which is to be expected according to the assumption that the rate of growth is proportional to the weight. The average relative rate of growth for the control groups was 4.78 per cent per day of the second period (10.5 to 15 days old) and 5.17 per day of the period from 6 to 15 days of age. Our experiments thus confirm the statement of Brody (1926–27, p. 646) that "fowl grows at 5 per cent per day up to 3 weeks."

TABLE I

Relative Growth Rate of Experimental Chicks According to Brody's Formula

Air temperature	Per cent growth per day: (100k) during the period of age of:		
	6–10.5 days	10.5–15 days	6–15 days
°C.			
21	6.2	6.9	6.5
27	5.7	6.6	6.2
32	5.8	6.4	6.1
38	4.9	4.4	4.6
40	3.8	4.6	4.2
40	3.5	5.5	4.5

(*b*) *Influence of Temperature on Food Consumption and Excretion Availability*

The increased rate of growth with the decrease in the temperature of the surrounding air is paralleled by an increased appetite. Table II contains the mean results of the food consumption per chick for the first and second period as well as for the entire time of the experiment.

Since the food consumption depends on the body size and the average weights are considerably different, it is advisable, for the comparison of the appetite at different temperatures, to reduce the data to comparable body size. The 3/4 power of body weight has been found to be most suitable as a basis for comparison of the metabolism of large and small animals (Kleiber, 1932). There is a close relation between metabolism and food consumption (Kleiber, 1933 *a*), therefore the 3/4 power of body weight should also be a suitable basis for comparing levels of food consumption.

TABLE II

Food Consumption, Excretion, and Availability at Various Temperatures
Grams Dry Matter per Day per Chick

Age	Air temperature, °C	21	27	32	38	40	40
days							
6–10	Food, *gm*	11.88	11.22	10.70	8.16	7.48	6.54
	Excretion, *gm*	4.72	4.34	3.95	2.97	2.78	2.52
	Available, *gm*	7.16	6.88	6.75	5.19	4.70	4.03
	Availability, *per cent*	60.3	61.3	63.1	63.7	62.8	61.3
11–15	Food, *gm*	18.18	15.38	12.27	9.30	8.40	9.36
	Excretion, *gm*	7.14	5.86	4.55	3.33	3.12	3.44
	Available, *gm*	11.04	9.52	7.72	5.97	5.28	5.92
	Availability, *per cent*	60.7	61.9	62.9	64.2	62.9	63.3
6–15	Food, *gm*	15.03	13.30	11.69	8.73	7.94	7.95
	Excretion, *gm*	5.93	5.10	4.32	3.15	2.95	2.98
	Available, *gm*	9.10	8.20	7.37	5.58	4.99	4.97
	Availability, *per cent*	60.6	61.7	63.0	64.0	62.8	62.5

The food consumption per $W^{3/4}$ in these trials is very nearly a linear function of the air temperature. The relation between F_t representing the intake of grams dry matter per unit of the 3/4 power of the body weight in kilos and the environmental temperature in degrees Centigrade may be expressed as follows:

$$F_t = 81.0 + 2.15\ (32.2 - t) \text{ for temperature below } 32.2°\text{C.}$$
$$F_t = 81.0 + 2.24\ (32.2 - t) \text{ for temperature above } 32.2°\text{C.}$$

It is surprising that this proportionality between temperature and food consumption should exist over such a considerable range of temperature. Since the maximum amount of food which can be taken in and digested per day must be limited, it was to be expected that toward the lower temperatures, the curve of the food consumption should approach asymptotically this maximal food capacity (see Fig. 1). More rapid decrease in food consumption with increased air temperatures above 38°C. was also expected. It seems to follow from the results of these experiments that at extremely high or low air temperatures, the food intake as a function of this temperature must change abruptly.

TABLE III

Liters CO_2 Produced per Day by Baby Chicks

Air temperature, °C		21	27	32	38	40	40
(a) Per chick							
1st period	6–10 days old	5.28	4.03	3.62	2.98	2.73	2.46
2nd period	11–15 days old	7.22	5.95	4.57	3.64	3.10	3.38
Total trial	6–15 days old	6.25	5.00	4.21	3.35	2.92	2.98
(b) Per kg. body weight							
1st period	6–10 days old	85.0	63.3	56.4	50.8	47.6	44.3
2nd period	11–15 days old	83.3	68.5	55.5	50.0	43.6	46.6
Total trial	6–15 days old	84.1	65.9	55.8	50.4	45.6	45.6
(c) Per kg.$^{3/4}$ of weight$^{3/4}$							
1st period	6–10 days old	42.4	31.8	28.5	25.1	23.3	21.5
2nd period	11–15 days old	44.4	37.3	29.8	26.0	22.6	24.2
Total trial	6–15 days old	43.4	34.5	29.3	25.6	22.9	23.0

The amount of dry matter consumed daily per kilo of body weight ranges from 120 gm. at 40°C. to 200 gm. at 21°C. If it is assumed that the body of the chick contains 30 per cent dry matter, it follows that at a temperature of 21°C. the chick eats daily 2/3 of the amount of dry matter in its own body. The increase in body weight per gram dry matter of food consumed shows a maximum of 0.375 gm. gain per gm. food at 32°C. dropping to 0.325 gm. at 21°C. and 0.324 gm. at 40°C.

The excreta of the chicks contain the non-digested part of the food as well as the secretion of the urinary system. They are therefore not comparable to the feces of mammals. One manifestation of this difference between the two kinds of excreta is the fact that the energy content as well as the C content per gram dry matter in the chick excreta are lower than the corresponding contents of the food. Calculated per 100 gm. dry matter, the food contained 41.2 gm. C, while the C content of the excreta varied from 34.3 gm. to 38.0 gm.

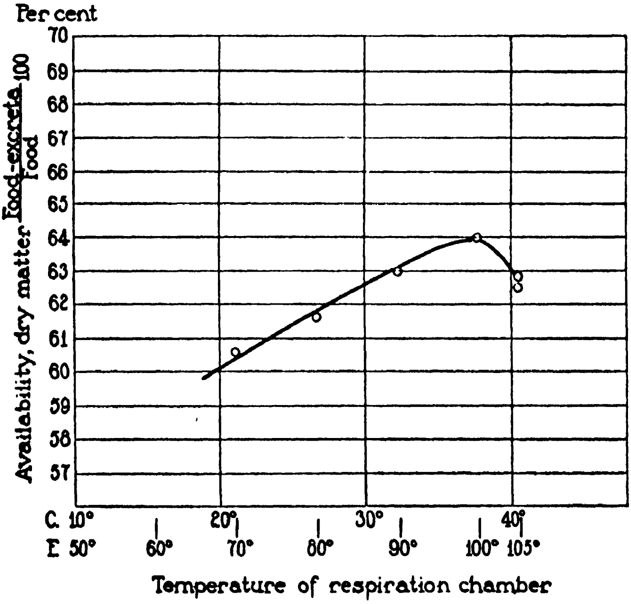

FIG. 4. Availability and air temperature.

with an average of 36.3 gm. The corresponding energy content of the feed was 432 Cal. while the excreta contained an average of only 371 Cal. with variations from 361 to 386 Cal. In herbivorous mammals, to the contrary, the energy and C are more concentrated in the feces than in the food, mostly due to the fact that the lignin, which has a high concentration of energy and carbon, is practically non-digestible. The nitrogen, on the other hand, is increased in the excreta of the chicks (4.15 gm. N per 100 gm. dry matter as compared to 2.89 gm. N per 100 gm. dry matter in the food).

We do not attempt to determine digestibility in these chick trials, but use for comparison a conception which is better adapted to nutrition work with birds and to which we give the name *availability* of the food. If per 100 gm. dry matter of food eaten, 40 gm. are excreted as feces and urine, 60 gm. dry matter remain *available* for combustion or for the production of body substance, then the availability of the dry matter is 60 per cent. The availability is given in Table II. The results for the first and second periods of each single trial are in very close agreement. The mean availability for both periods is plotted against the environmental temperature in Fig. 4.

At an air temperature of 38°C., the availability is 64 per cent, the maximum in our experiments. With decreasing temperature it drops at the average rate of 0.12 per cent per degree drop in temperature.

TABLE IV

Respiratory Quotient of Full Fed Baby Chicks at Different Temperatures

Air temperature, °C.	21		27		32		38		40		40	
Age	Day	Night	Day	Night	Day	Night	Day	Night	Day	Night	Day	Night
days												
6–10	0.994	0.914	1.036	0.940	1.053	0.965	1.040	0.998	1.004	0.869	0.984	0.865
11–15	1.011	0.936	1.157	0.916	1.051	0.906	1.039	0.957	1.061	0.911	1.070	0.966
6–15	1.002	0.925	1.080	0.928	1.052	1.928	1.040	0.977	1.032	0.890	1.032	0.915
Day and night.....	0.971		1.018		0.990		1.012		0.966		0.979	

At temperatures above 38°C. the availability is also decreased to an average of 62.7 per cent at 40°C. It remains to be studied whether the drop in the availability with decreasing temperatures is the result of the increasing amount of food consumed, which would parallel unpublished results obtained by the senior author on rabbits and sheep, which show a decrease in the digestibility with an increase in food consumption.

(*c*) *Influence of the Environmental Temperature on the Respiratory Exchange*

Like other homoiotherms, the baby chicks respond to a lowering of the environmental temperature with an increased metabolism. This behavior is clearly shown in Table III.

At 40°C. one chick of 6 to 15 days of age produced a daily average of 2.95 liters CO_2. With every drop in temperature, without exception, the average daily CO_2 production was increased. At 21°C. the respiratory exchange was twice as high as that at 40°C., reaching a level of 6.25 liters CO_2. The same behavior is shown if the daily CO_2 production is calculated separately for the first and second periods of the experiment. Essentially the same result is obtained if the CO_2 production is calculated per kilo of body weight. It ranges, for the total period (6 to 15 days of age), from 45.6 liters CO_2 per kilo at 40°C. to 84.1 liters CO_2 per kilo at 21°C., where the daily loss of 165 gm. of CO_2 per kilo of body weight amounts to more than 50 per cent of the dry matter contained in the body of the chick.

Calculated to the 3/4 power of the body weight, the daily CO_2 production of the chicks increased from 23 liters CO_2 per $kilo^{3/4}$ at 40°C. to 43.4 liters per $kilo^{3/4}$ at 21°C. At 38°C. the chicks produced daily 25.6 liters CO_2 per $kilo^{3/4}$.

In four beef heifers in another experiment, the daily CO_2 production at full feed amounted to 24.2 liters per unit of the 3/4 power of the body weight. Since the temperature of 20°C., at which the heifers were kept, corresponds better, physiologically, to 38°C. for baby chicks than to a lower temperature, the comparison seems to indicate that the respiratory exchange of our baby chicks was in agreement with the rule that the metabolism per unit of the 3/4 power of the body weight of warm blooded animals is independent of body size.

During the experiment, the daily CO_2 production per unit of the 3/4 power of the body weight increased at an average daily rate of 1.3 ± 0.5 per cent of the mean CO_2 production for the total period of 9 days.

This increase in the respiratory exchange with increasing age is somewhat comparable to the increase in metabolism of children. According to the measurements of Benedict and Talbot (1921) the basal metabolism of boys increases during the period of 6 months to 18 months of age 14 per cent of the value of the age of 1 year. The corresponding increase for girls is 15 per cent. If it can be assumed that the metabolism of the chicks at full food is a certain multiple of the basal metabolism, then it would follow that the per cent increase in metabolism in children in 1 year is of the same magnitude as that of the baby chicks in 12 days. The ratio of 1 year human life to 12 days chick life seems to be of the same order for sex maturity.

The *respiratory quotient* varies little for different temperatures, as shown in Table IV. There seems to be a tendency for the R.Q. to be lower at the extremely low and high air temperatures. Much more marked than the variation at different temperatures is the variation in R.Q. between day and night. It seems natural to explain this difference by the fact that the chicks ate no food during the night. The average R.Q. of all the day runs is 1.033 ±0.010, and the average of the night runs 0.924 ±0.010. The fact that the R.Q. during the day is higher than unity may be related to an intensive production of body fat from carbohydrates. During the night, the chicks were without food and their lower R.Q. indicated a decrease in fat production. The metabolism of the birds during the night, however, was far from fasting metabolism, since 24 to 36 hours after the last food the chicks had a respiratory quotient of 0.734 ±0.009 (Table VII).

(*d*) *N*, *C*, *and Energy Balances*

For the sake of economy of space the twenty-seven tables giving the N, C, and energy balances are not printed. From the composition of the food and feces, and the amount of dry matter eaten and excreted daily, the amount of N, C, and energy taken in and excreted daily is calculated. The difference between the intake and excretion is the amount of N, C, and energy which is available for combustion or production of body substance. The amount of C lost as CO_2 is calculated from the result of the respiration trial. For each liter CO_2 given off by the animal, 0.5359 gm. C is subtracted from the available C. The rest is the carbon stored in the body (net). The amount of protein gained is calculated by multiplying the figure for the available N by 6.25. The amount of C contained in the stored protein is 3.25 times the amount of available N. The amount of C in protein is subtracted from the total amount of C stored, and the rest is the amount of C in the produced body fat, which contains 76.5 per cent C. The net energy is obtained as the sum of the heat of combustion of the stored protein (1 gm. protein = 5.7 Cal.)[5] and the stored fat (1 gm. body fat = 9.5 Cal.). The heat production of the animal is calculated as the difference between the available energy and the net energy.

The heat production of the birds was larger in the second period of the trial, corresponding to the increased respiratory exchange which has already been discussed. The influence of the environmental temperature on the heat production is very consistent. In both periods, the heat production of the animal was greater the lower the air temperature. Table V gives the summary of the results on energy metabolism obtained per chick for the total duration of the experiment.

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[5] In this paper the energy is expressed throughout in kilogram calories.

As the temperature is increased above 32°C. the heat production is decreased at the same rate as the food consumption. At the extremely low temperatures, the food consumption seems no longer to keep pace with the increase in metabolism. At 21°C. the food intake was increased to 128 per cent, the available energy to 121 per cent, the heat production, however, to 153 per cent of the value at 32°C. This observation seems to confirm the hypothesis that with lower temperature, the heat production tends to approach the energy intake, since the latter is naturally limited.

TABLE V

Energy Transformation in Full Fed Baby Chicks for the Period of 6 to 15 Days of Age

Air temperature, °*C*	21	27	32	38	40	40
Average body weight, *gm*	74.2	75.4	71.7	65.1	64.0	64.5
Average units of $W^{3/4}$, $kg.^{3/4}$	0.142	0.144	0.138	0.129	0.127	0.128
Energy in food per day per chick, *Cal*	65.2	56.6	51.1	37.5	34.1	34.5
Energy in excreta per day per chick, *Cal*	22.9	18.8	16.1	11.4	10.8	11.1
Available energy per day per chick, *Cal*	42.3	37.8	35.0	26.1	23.3	23.4
Heat production per day per chick, *Cal*	35.4	27.4	23.2	17.4	15.9	17.4
Net energy per day per chick, *Cal*	6.9	10.4	11.8	8.7	7.4	6.0

The net energy shows a maximum of 11.8 Cal. per chick per day at 32°C. decreasing to 6.9 Cal. at 21°C. and an average in two trials of 6.7 Cal. at 40°C. The energy transformation of the baby chicks as a function of the air temperature is illustrated in Fig. 5. In order to avoid in this graph possible influences of body size, the energy exchange is calculated per unit of the 3/4 power of body weight. The energy intake appears to decrease in proportion to the increase in the air temperature at a rate of -10.4 Cal. per °C. The available energy, however, shows the tendency to reach a maximum at low environmental temperatures. It seems to follow from these two curves that in baby chicks the expected limitation of energy intake is a question of the digesting or absorbing power of the intestinal tract rather than of the appetite. The curve for the heat production of the animals is bent in the opposite direction to that for the available energy. By extrapolation of these two curves a point of coincidence is found between 15°C. and 20°C. At this temperature the animal would lose as much energy in the form of heat as it can make available from the food; therefore, no body substance could be produced. The net energy at this low temperature would consequently be zero. It reaches its maximum at 32°C. and drops as the temperature of the environment approaches the body temperature which, according to several measurements after the trial, was 41.7°C.

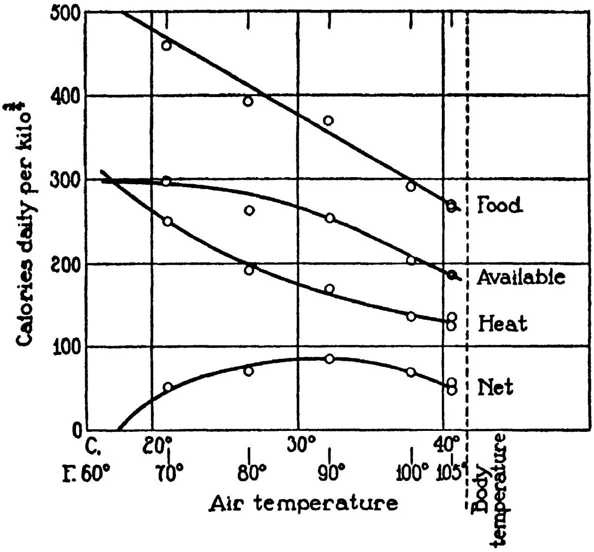

FIG. 5. Energy exchange and air temperature.

(e) *Composition and Energy Content of the Gain in Body Substance*

The energy metabolism of our chicks has been calculated on the basis of the C and N balances assuming that the main organic constituents of the gain in body substance are protein and fat. Table VI shows the influence of environmental temperature on the daily production of these two groups of compounds. The storage of protein was higher, as was the gain in body weight, the lower the outside temperature. The production of body fat, on the other hand, had a maximum at 32°C. air temperature, falling almost to zero as the temperature decreased to 21°C. and also dropping considerably with increasing temperatures above 32°C. The daily gain in net energy by no means paralleled the growth rate (in terms of body weight). At 21°C. air temperature with the highest gain in weight, the storage of net energy was next to the lowest obtained, the maximum being at 32°C. This result shows that the body weight is no general criterion for the effect of food on energy storage in the animal.

The question may be raised whether the gain in weight or the gain in energy is a more adequate expression for growth. If the deposition of body protein is considered an important characteristic of growth, then the gain in weight would be preferred to the gain in energy. It is seen in Table VI that the gain in protein changes only from 0.22 gm. to 0.27 gm. per gm. increase in body weight. No influence of the environmental temperature on the protein content of the gain in body weight seems to exist. Such an influence on the gain in body fat per unit of gain in weight is, however, very marked. The greatest gain of fat was made at a temperature of 32°C., 0.153 gm. fat being deposited per gram increase in weight. The increase in weight at 21°C. contained only 1 per cent fat and consequently a higher water content (76.3 per cent compared with 58.6 per cent at 38°C.). The gain at 38°C. had the highest concentration of energy, 2.93 Cal. per gm. of increase in weight, compared to 1.41 Cal. at 21°C.

TABLE VI

Composition and Energy Content of the Daily Gain of Baby Chicks at Various Environmental Temperatures

Period: 6 to 15 Days of Age

Air temperature, °*C*	21	27	32	38	40	40
Daily gain in body weight, *gm*	4.88	4.64	4.39	2.97	2.74	3.09
Daily production of body protein, *gm*	1.10	1.08	0.97	0.79	0.69	0.67
Daily production of body fat, *gm*	0.06	0.44	0.67	0.44	0.37	0.24
Daily net energy, *Cal*	6.9	10.4	11.8	8.7	7.4	6.0
Per gm. increase in body weight:						
Body protein, *gm*	0.225	0.233	0.221	0.266	0.252	0.217
Body fat, *gm*	0.012	0.095	0.153	0.148	0.135	0.078
Inorganic matter (mostly water), *gm*	0.763	0.672	0.626	0.586	0.613	0.705
Energy, *Cal*	1.41	2.24	2.69	2.93	2.70	1.94

(*f*) *Basal Metabolism*

The basal metabolism of the chicks has been determined at the end of each trial, starting in the evening when the chicks had been without food for 24 hours, and ending the following morning. The results of these experiments, calculated to 24 hours are given in Table VII. The heat production is calculated from the oxygen consumption, using a standard of 4.7 Cal. per liter of oxygen consumed. The basal heat production is increased as the outside temperature is decreased. It is to be expected in warm blooded animals that above a certain so called critical temperature of the environment, the metabolism is independent of changes in this temperature. No evidence of such a critical temperature was found. Considering the relatively large variations, the number of experiments is not adequate to conclude that no such critical temperature exists for the metabolism of these young birds. If a critical temperature exists it is safe to state on the basis of our trials that it is considerably higher for baby chicks than adult chickens which Mitchell and Haines (1927 *a*) found to be 16.7°C.

TABLE VII

Basal Metabolism of Baby Chicks at Various Temperatures at 16 Days of Age

Air temperature	No. of experiment	Body weight W	$W^{3/4}$ kg.$^{3/4}$	Daily respiratory exchange per chick		R.Q. $\left(\frac{CO_2}{O_2}\right)$	Daily heat production		
				CO_2	O_2		Per chick	Per kg.	Per $W^{3/4}$
°C.		gm.		liters	liters		cal.	cal.	cal
21	11	85.6	0.158	3.28	4.32	0.76	20.3	237	128
27	7	88.6	0.162	2.50	3.52	0.71	16.5	186	102
32	9	84.3	0.156	2.14	3.01	0.71	14.1	167	90
38	6	73.1	0.140	1.81	2.48	0.73	11.7	160	83
40	8	73.9	0.142	1.12	1.53	0.73	7.2	98	51
40	12	74.4	0.142	1.50	2.11	0.71	9.9	133	70

The average figure for the basal metabolism of our chicks at 38°C. and 40°C. is 68 Cal. per kilo$^{3/4}$. From the measurements of Mitchell and Haines (1927 *b*) it follows that the basal metabolism of their mature hens amounted to an average of 64 Cal. per kilo$^{3/4}$. The corresponding figures per unit of weight are 130 Cal. per kilo for the baby chicks and 54 Cal. per kilo for the mature hens. This comparison shows that the metabolism of fowls of different size is more closely related to the unit of the ¾ power of the weight than to the unit of weight itself, which is in accordance with the results in other homoiotherms as discussed in an earlier paper (Kleiber, 1932).

(*g*) *The Heat Increment of the Feed at Various Temperatures*

From the figures for the basal metabolism and the metabolism at full feed the heat increment (Armsby) or specific dynamic action (Rubner) of the feed has been calculated in Table VIII. For this calculation, it had to be considered that during the test for the basal metabolism the birds were heavier and older than in the average during the period of full feed. In order to eliminate the discrepancies in weight, the heat production in both cases is calculated per unit of the 3/4 power of the body weight.[6]

TABLE VIII

Heat Increment of the Food of Baby Chicks at Various Environmental Temperatures

Temperature of air, °C	21	27	32	38	40	40
Daily basal heat production per kg.$^{3/4}$ calculated to the age of 10.5 days, *Cal*	121	97	85	78	48	66
Daily heat production at full feed, per kg.$^{3/4}$, *Cal*	249	190	168	135	125	136
Daily heat increment of full feed per kg.$^{3/4}$, *Cal*	128	93	83	57	77	70
Daily available energy in food per kg.$^{3/4}$, *Cal*	298	262	254	202	183	183
Heat increment per 100 Cal. available. Energy at full feed, *per cent*	43	36	33	28	42	38

For making the basal metabolism determined at the age of 16 days comparable to the metabolism at full feed with an average age of 10.5 days, it has been assumed that during the period studied the basal metabolism in these birds increased 1 per cent of the average for each day increase in age as found for the metabolism at full feed (see page 714). This assumption seems to be justified because the total energy intake for very different animals seems to approximate the same multiple of the basal metabolism (Kleiber, 1933*a*), and the metabolism at full feed is closely related to the energy intake. Thus a theoretical basal metabolism for the age of 10.5 days has been calculated on the basis of the equation:

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[6] For the sake of simplicity, the average metabolism per chick as given in Table V has been divided by the 3/4 power of the average weight. The result does not differ considerably from that obtained by calculating the metabolism for each day to the unit of the 3/4 power of the corresponding weight and then taking the average.

$$B_{10.5} \times (1 + 0.01 \times 5.5) = B_{16}$$

where $B_{10.5}$ = basal metabolism at the age of 10.5 days,

and B_{16} = basal metabolism at the age of 16 days.

According to Rubner's compensating theory it is to be expected that at lower environmental temperatures the specific dynamic action is decreased because the extra heat developed after food consumption is used for maintaining the body temperature and saves a corresponding amount of food or body substance which would have been used as fuel. Consequently, the gap between basal metabolism and metabolism at full food should become smaller as the outside temperature decreases and if it falls below the critical temperatures for maximal food intake, the heat production should become independent of the amount of food consumed, in other words, the specific dynamic action should disappear (see Fig. 1).

The results shown in Table VIII are not in accordance with this theory. Although 21°C. is considered an extremely low temperature for baby chicks, the specific dynamic action of the food at this temperature is higher than at 32°C. and 38°C. not only in absolute terms but also in per cent of the available energy of the food consumed.

This result may be explained partly by the possibility that the chicks can decrease their heat requirement by huddling together. It has been observed in the trial at 27°C. but particularly at 21°C. that when the chicks are not eating, they gather in a corner of the cage, forming a pyramid. This huddling of the chicks at low temperature decreases their heat loss 15 per cent (Kleiber and Winchester, 1933). It is to be regarded as a third kind of temperature regulation which we have termed social temperature regulation. It tends to make the basal metabolism at low temperature lower than it would be without this huddling and consequently increases the gap between basal metabolism and metabolism at full feed.

(*h*) *Efficiency of Energy Transformation at Various Temperatures*

By efficiency of food utilization may be understood the ratio of an increase in net energy to the increase in food necessary to produce this net energy

$$\eta_p = \frac{\Delta A}{\Delta U}$$

η_p may be classified as partial efficiency if ΔA is a difference in net energy and ΔU the corresponding difference in food energy. Food energy may be taken as the energy of the food intake, the digested energy, or the available energy.

TABLE IX

Partial Efficiency for Growth in Baby Chicks at Various Temperatures

Air temperature, °*C*	21	27	32	38	40	40
Net energy per 100 Cal. of available food energy	57	64	67	72	58	62

On the other hand, efficiency may also mean the ratio of the total net energy to the total food energy.

$$\eta_t = \frac{A}{U}$$

In contradistinction to the partial efficiency, η_t may be called the *total efficiency*. The partial efficiency is always positive. Recent results show that it varies with the plane of nutrition, being somewhat higher at low levels.[7] It is positive also in undernutrition, as a decrease in the loss of body substance due to an increase in food consumption may be taken as an increase in net energy. The determination of the partial efficiency requires the measurement of the metabolism at two different food levels. One level may be at zero. Thus, in the case of the chicks in the experiments discussed here, the basal metabolism and metabolism at full feed are used. The increase in net energy in this sense is the difference between the increase of the available energy and the heat increment. Thus Table IX is calculated from Table VIII.

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[7] In an experiment of Forbes *et al.* (1930) the partial efficiency dropped from 87 to 64 per cent of the metabolizable energy as the plane of nutrition was increased from ½ maintenance to 3 times maintenance. Wiegner and Ghoneim (1930) applied Mitscherlich's law to the partial efficiency of food utilization in animals.

The partial efficiency is subject to a relatively large experimental error. From Kellner's data (Kellner and Köhler, 1900) it may be calculated that the standard error for the partial efficiency of starch for fattening adult steers is ±11 per cent of the mean. The deviation between the results of the two chick trials at 40°C. is ±6.7 per cent of the mean. The increase in efficiency from 57 per cent at an air temperature of 21°C. to 72 per cent at an air temperature of 38°C., a difference of 23 per cent of the mean, appears thus to be barely significant.

TABLE X

Total Efficiency of Energy Utilization in Growing Baby Chicks at Various Temperatures

Air temperature, °C	21	27	32	38	40	40
Total efficiency $\left(\frac{\text{Net energy} \times 100}{\text{Available food energy}}\right)$	16	28	34	33	32	26

The total efficiency is related to the partial efficiency according to the following equation:

$$\eta_t = \frac{A}{U} = \frac{\eta_p(U - E)}{U} = \eta_p\left(1 - \frac{E}{U}\right)$$

where

η_t = total efficiency
η_p = partial efficiency
U = energy of food intake
E = energy of food for maintenance.

At a given partial efficiency the total efficiency increases with increasing plane of nutrition. It becomes zero when the total food energy is equal to the energy necessary for maintenance.

Since the total efficiency in our trials is determined independently of the basal metabolism, the influence of the social temperature regulation on the basal heat production does not affect the results of the total efficiency. Therefore, these results calculated from Table V are less questionable than those on the partial efficiency.

The total efficiency reaches its maximum at 32°C. The environmental temperature had a more pronounced influence on the total efficiency than on the partial efficiency. 16 per cent total efficiency at 21°C. is only 47/100 of the maximum total efficiency at 32°C., whereas 57 per cent partial efficiency at 21°C. is 79/100 of the maximum partial efficiency at 38°C.

DEDUCTION

From general considerations it has been concluded that an optimal environmental temperature for the conversion of food energy to the energy of body substance should exist not only for cold blooded but also for warm blooded animals. At extremely low environmental temperature all the energy which an animal is able to absorb is used as heat for maintaining the body temperature at a constant level; at extremely high outside temperature theoretically the animal's appetite is decreased to such an extent that the energy intake does not exceed the maintenance requirement.

In our experiments with baby chicks we did not reach either of these extremes. We found that within the range of temperatures covered in our investigation the total food intake was a linear function of the environmental temperature and not a curve as suggested in Fig. 1. A curve of the expected type has, however, been obtained for the intake of metabolizable energy due to the limitation of the absorbing power of the intestinal tract. At high environmental temperature the appetite was decreased. In accordance with the preliminary hypothesis the heat production of the animals tended to approach the intake of metabolizable energy at high as well as at low environmental temperature. The prediction of an optimal environmental temperature for the efficiency of energy utilization is thus supported by the results of our trials.

SUMMARY

1. An optimum of environmental temperature is to be expected for the utilization of food energy in warm blooded animals if their food intake is determined by their appetite.

2. Baby chicks were kept in groups of five chicks in a climatic cabinet at environmental temperatures of 21°, 27°, 32°, 38°, and 40°C. during the period of 6 to 15 days of age. The intake of qualitatively complete food was determined by their appetite. Food intake, excretion, and respiratory exchange were measured. Control chicks from the same hatch as the experimental groups were raised in a brooder and were given the same food as the experimental chicks. The basal metabolism of each experimental group was determined from 24 to 36 hours without food at the age of 16 days.

3. The daily rate of growth increased with decreasing environmental temperature from 2.74 gm. at 40°C. to 4.88 gm. at 21°C. This was 4.2 to 6.5 per cent of their body weight.

4. The amount of food consumed increased in proportion to the decrease in temperature.

5. The availability of the food, used for birds instead of the digestibility and defined as $\frac{\text{Food-excreta}}{\text{Food}}$ showed an optimum at 38°C.

6. The CO_2 production increased from 2.95 liters CO_2 per day per chick at 40°C. to 6.25 liters at 21°C. Per unit of the 3/4 power of the body weight, 23.0 liters CO_2 per $\text{kilo}^{3/4}$ was produced at 40°C. and 43.4 liters per $\text{kilo}^{3/4}$ at 21°C. The CO_2 production per unit of 3/4 power of the weight increased at an average rate of approximately 1 per cent per day increase in age. The R.Q. was, on the average, 1.04 during the day and 0.92 during the night.

7. The net energy is calculated on the basis of C and N balances. A maximum of 11.8 Cal. net energy per chick per day was found at 32°C. At 21°C. only 6.9 Cal. net per day per chick was produced and at 40°C. an average of 6.7 Cal.

8. The composition of the gained body substance changed according to the environmental temperature. The protein stored per gram increase in body weight varied from 0.217 to 0.266 gm. protein and seemed unrelated to the temperature. The amount of fat per gram gain in weight dropped from a maximum of 0.153 gm. at 32°C. to 0.012 gm. at 21°C. and an average of 0.107 gm. at 40°C. The energy content per gram of gain in weight had its maximum of 2.95 Cal. per gm. at 38°C. and its minimum of 1.41 Cal. per gm. at 21°C. at which temperature the largest amount of water (0.763 gm. per gm. increase in body weight) was stored.

9. The basal metabolism increased from an average of 60 Cal. per $\text{kilo}^{3/4}$ at an environmental temperature of 40°C. to 128 Cal. per $\text{kilo}^{3/4}$ at 21°C. No indication of a critical temperature was found.

10. The partial efficiency, *i.e.* the increase in net energy per unit of the corresponding increase in food energy, seemed dependent on the environmental temperature, reaching a maximum of 72 per cent of the available energy at 38°C. and decreasing to 57 per cent at 21°C. and to an average of 60 per cent at 40°C.

11. The total efficiency, *i.e.* the total net energy produced per unit of food energy taken in, was maximum (34 per cent of the available energy) at 32°C., dropped to 16 per cent at 21°C., and to an average of 29 per cent at 40°C.

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THE ACCUMULATION OF ELECTROLYTES

VI. THE EFFECT OF EXTERNAL pH

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It would be natural to suppose that potassium enters *Valonia* as KCl since it appears in this form in the sap. We find, however, that on this basis we cannot predict the behavior of potassium in any respect. But we can readily do so if we assume that it penetrates chiefly[1,2] as KOH. We may then say that under normal conditions potassium enters the cell because the ionic activity product (K) (OH) is greater outside than inside. This hypothesis leads to the following predictions:

1. When the product (K) (OH) becomes greater inside (because the inside concentration of OH^- rises, or the outside concentration of K^+ or of OH^- falls) potassium should leave the cell, though sodium continues to enter. Previous experiments,[3] and those in this paper, indicate that this is the case.

2. Increasing the pH value of the sea water should increase the rate of entrance of potassium, and *vice versa*. This appears to be shown by the results described in the present paper.

The experiments were made in Bermuda on *Valonia macrophysa*, Kütz. The technique, unless otherwise stated, was that previously employed.[3]

Methods

The cells, carefully seasoned in the laboratory, were exposed to sea waters with higher and lower hydrogen ion activities than normal for periods up to

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[1] *Cf.* Osterhout, W. J. V., *Biol. Rev.*, 1931, **6**, 369; *Ergebn. Physiol.*, 1933, **35**, 967.

[2] Regarding the situation in land plants see Osterhout, W. J. V., *Science*, 1912, **36**, 571.

[3] Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 301; 1931–32, **15**, 537.

15 days, but care was taken to prevent injury by limiting the magnitude of the pH changes. The temperature fluctuated between 15° and 25°C., but did not vary more than 5°C. in one experiment.

In the preliminary experiments it was found that the pH could not be lowered much below 6.5 without producing obvious signs of injury in 24 hours. The upper limit which the cells would tolerate was not determined. It is undesirable to go above pH 9.4 on account of the rapid precipitation of calcium carbonate. We worked at pH 8.8 which the cells could tolerate for many days without injury.

It was found that the pH of sea water in contact with the cells tends to change so rapidly as seriously to vitiate the conclusions from experiments where no account of this was taken. Hence the experiments were carried out in such a way as to minimize the change (or else to exaggerate it).

No single procedure for exposing the cells was used. In some cases tubes and glass gutters (as described previously) were employed,[3] in others stoppered bottles of Pyrex glass.

Sometimes the cells were exposed out of doors with the usual alternations of light and dark, and sometimes inside under constant artificial illumination. In some cases there was a current of sea water, in others it was changed infrequently. The particular method of exposure will be described in detail under each experiment as well as the reason for the procedure. In every experiment 16 liters of sea water at each pH were prepared and in the experiment with circulation it was passed over the cells again and again.

Growth was measured on a sample lot of cells believed to be representative of the whole as described elsewhere;[3] the apparatus for measuring volume was that described in a previous paper.[3]

The pH of the sea waters was determined as described in a previous paper, brom thymol blue, cresol red, and thymol blue being used for the low, normal, and high pH sea waters respectively. For sap chlor phenol red was used.

The analyses for potassium, sodium, and halide were also carried out as described in the papers cited above. Since sodium is determined by difference the figures are less accurate than in the other cases. This should be borne in mind in those cases where the tables show an apparent loss in moles of sodium.

Each analysis required the sap of from 50 to 75 cells: hence the analyses represent the average of this number of cells. Absence of any appreciable increase in the sulfate during exposure was taken to indicate absence of severe injury. The cells were examined macroscopically and microscopically before and after exposure and appeared to be in excellent condition.

The usual precautions were taken to obtain representative samples of sap for analysis. In most of the experiments large numbers of cells were available so that no large ones (*i.e.* over 0.5 cc.) had to be used.

As is well known, sea water is reasonably well buffered by the presence of sodium bicarbonate at a concentration of about 0.002 normal and of free CO_2

at a partial pressure of about 0.0003 atmosphere, which means that it is practically in equilibrium with the atmosphere.

If it is desired to change the pH of the sea water three courses are open. (*a*) The bicarbonate-carbonic acid buffer system may be replaced by another in which the weak acid is not in equilibrium with the air. (*b*) The concentration of bicarbonate ion may be kept constant and the CO_2 tension raised or lowered to lower or raise the pH. (*c*) The CO_2 tension may be kept constant and the concentration of sodium bicarbonate and sodium carbonate raised or lowered to raise or lower the pH.

Alternative (*a*) would, in theory, overcome the difficulty associated with the change in the CO_2 tension of the sea water caused by the photosynthetic and respiratory processes of the cells. However, it would also deprive the cells of free CO_2. It might be difficult also to maintain the air over the sea water free of CO_2 during long experiments. Nevertheless if only part of the bicarbonate-carbonic system were replaced, it seemed possible that the cells might receive their normal supply of CO_2 and at the same time the sea water pH might remain more constant if there were present a buffer system not sensitive to photosynthesis or respiration. Preliminary experiments were performed to test this possibility, using phosphoric and succinic acids. In each case 9/10 of the bicarbonate was replaced. In the case of phosphoric acid at all pH values studied the resulting sea waters were toxic in 24 hours. Succinic acid on the other hand was well tolerated, but in comparison with a control with normal sea water there was no decided improvement in the constancy of the pH.

Alternative (*b*) was considered to be impractical for long experiments because of the difficulty of maintaining over the sea water an atmosphere containing more or less CO_2 than the normal amount.

Alternative (*c*) was therefore adopted as the most practical. Moreover, our experiments on the penetration of CO_2 into *Valonia*[4] show that the pH of the sap is strongly influenced by changes in the concentration of free CO_2 in the sea water. Hence the plan in which the sea water used is in equilibrium with normal air is to be preferred, because we know within limits the pH to be expected in the sap of cells in normal sea water under these conditions.

When the pH of sea water is to be lowered it would seem possible to calculate the amount of HCl required to be added to decompose enough bicarbonate to produce the needed lowering. Actually this can rarely be done. This may be due to the presence in the sea water of varying amounts of finely divided $CaCO_3$. The sea water used in these experiments was obtained about ½ mile from land in the 30 foot ship channel where it was frequently slightly milky, especially for several days after storms which in winter in Bermuda often stir up the coral sand at the bottom. In large bottles in the laboratory it appeared perfectly clear.

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[4] Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, **9**, 255. Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 695.

Our method of procedure for lowering the pH of the sea water was to add sufficient HCl to lower the pH below the required point. Air (previously saturated with moisture over sea water) was then bubbled through until the pH rose above the required point. Hereafter further additions of HCl were made with prolonged aeration in between until at length the required pH was reached and did not change with further aeration. This procedure was necessitated by the fact that the sea water easily becomes supersaturated with respect to CO_2 when acidified and it requires prolonged aeration to adjust this.

When the required point had been reached a sample of sea water was analyzed for total CO_2, using the Van Slyke constant volume apparatus. It was found that when the pH remained constant at 6.8–7.0 after aeration the concentration of CO_2 in the sea water was in the neighborhood of 0.00011 M. This agrees roughly with McClendon's[5] results assuming that the CO_2 tension of the atmosphere is in the neighborhood of 0.0003.[6]

In raising the pH above normal, 0.6 N NaOH was added in small amounts with prolonged aeration with air saturated over sea water between additions until the required pH was reached. When the pH was raised above 9.4 it was found that $CaCO_3$ was slowly precipitated, but by working in the manner described the loss of calcium due to this was reduced to a minimum. It was found that in the course of the experiments photosynthesis raised the pH above 9.4 and as a result calcium was lost. In order to determine the extent of this loss the high pH sea water was analyzed for total CO_2 before and after the experiment in some cases. In the worst case the loss of total CO_2, presumably as $CaCO_3$, amounted to about 0.001 equivalent per liter, and since the original concentration of calcium in normal sea water is 0.02 normal it follows that the loss was only about 5 per cent, which we do not consider enough to vitiate the results seriously.

RESULTS

A. At High pH: Flowing Sea Water

To keep the pH as constant as possible sea water was made to flow over the cells (day and night) as described in previous papers.[3,7] The cells were kept out of doors and shaded from direct sunlight.

In each experiment one lot was exposed to sea water at pH 8.8 and the other to a control in sea water at 8.2. The results are given in Tables I, II, and III (see also Fig. 1).

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[5] McClendon, J. F., *J. Biol. Chem.*, 1917, **30**, 274.

[6] After completing the experiments our attention was called to the paper by Tomita (Tomita, G., *J. Shanghai Sc. Inst., Sect. IV*, 1933, **1**, 19) in which he calls attention to the importance for biological work of adjusting the CO_2 tension of sea water after acidification.

[7] In the present experiments about a liter and a half per hour flowed over the cells which were spread out in a thin layer. The CO_2 was replaced by frequent aeration.

It is evident that the rate of growth and of entrance of potassium was greater at the high than at the normal pH. The gain in moles[8] at pH 8.8 is compared with that at pH 8.2 in Table III, and the calculated gain is also given. The calculation is as follows. Assuming that the rate of entrance is proportional to $(K_o)(OH_o) - (K_i)(OH_i)$ we may write[9] as an approximation for the first period in Table I

TABLE I

Analytical Data on Saps of Valonia

Section A, Experiment 1

Nominal pH of sea water	Time	pH of sap	Molecular concentrations				Ratio K ÷ Na	Volume*	Gram moles × 10^3 = moles per liter × volume			
			K	Na	K + Na	Halide			K	Na	K + Na	Halide
	days							*ml.*				
8.8 (High)	0	6.15	0.4952	0.1408	0.6360	0.6275	3.52	8.73	4.323	1.229	5.552	5.478
	3	6.10	0 4961	0.1388	0.6349	0.6263	3.57	9.17	4.550	1.273	5.823	5.744
	6	6.30	0 4986	0.1322	0.6308	0.6287	3.77	9.41	4.692	1.244	5.936	5.916
	10	6.10	0.4930	0.1415	0.6345	0.6354	3.48	9.68	4.790	1.369	6.159	6.150
	15	5.95	0.4898	0.1419	0.6317	0.6299	3.45	9.93	4.864	1.408	6.272	6.255
8.2 (Control)	0	6.15	0.4952	0.1408	0.6360	0.6275	3.52	8.73	4.323	1.229	5.552	5.478
	3	5.95	0.4944	0.1431	0.6375	0.6293	3.45	8.91	4.406	1.275	5.681	5.608
	6	6.10	0.4817	0.1477	0.6294	0.6202	3.21	9.14	4.403	1.350	5.753	5.669
	10	6.00	0.4826	0.1525	0.6351	0.6244	3.16	9.31	4.493	1.420	5.913	5.813
	15	5.90	0.4779	0.1518	0.6297	0.6250	3.16	9.43	4 508	1.432	5.940	5.894

* In order to make the volumes the same for both lots at the start the values for pH 8.8 were multiplied by 8.73 ÷ 8.52 and those for the control by 8.73 ÷ 8.76 (see Table V).

$$\frac{\text{Rate at pH 8.8}}{\text{Rate at pH 8.2}} = \frac{0.012\,(10^{-5.2}) - 0.4952\,(10^{-7.85})}{0.012\,(10^{-5.8}) - 0.4952\,(10^{-7.86})} = 5.72$$

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[8] It is obvious that the rate of entrance must be measured by the gain in moles since it cannot be inferred from the concentration owing to the entrance of water.

[9] This can only be regarded as an approximation. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529; Osterhout, W. J. V., Kamerling, S. E., and Stanley, W. M., *J. Gen. Physiol.*, 1933–34, **17**, 445, 469.

The subscripts *o* and *i* refer to activities outside and inside respectively.

Since the ionic strength is about the same inside and outside it is permissible

Table III also gives calculations for ionic exchange (for which see p. 747).

The rate of entrance of potassium does not increase so much as

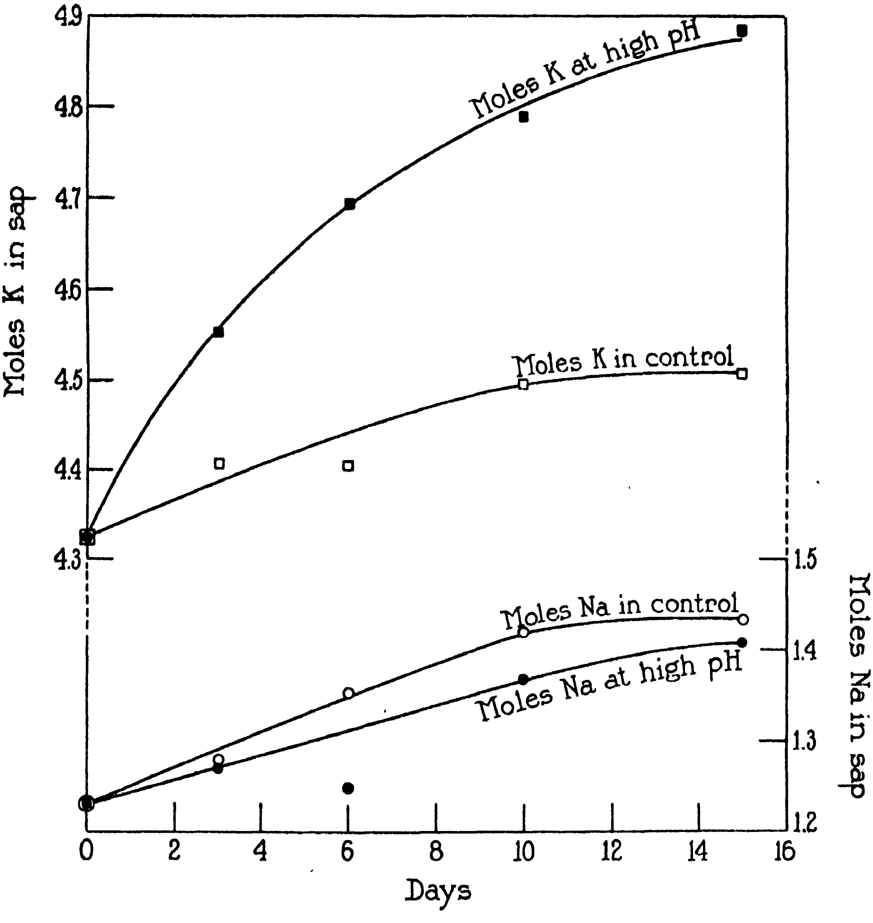

FIG. 1. Moles of potassium and of sodium entering *Valonia* at high pH (about 8.8) and in a control experiment where the pH just outside the protoplasm started at about 8.2 and rose somewhat: it apparently did not rise as high as in the other lot and in consequence potassium entered less rapidly (the pH was kept down by a rapid flow of sea water). Section A, Experiment 1. The curves are drawn free-hand to give an approximate fit.

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for comparative purposes to use concentrations in place of activities (*cf.* Zscheile, F. P., Jr., ***Protoplasma***, 1930, **11**, 481).

TABLE II

Analytical Data on Saps of Valonia
Section A, Experiment 2

Nominal pH of sea water	Time	Volume*	Adjusted volume†	pH of sap	Molecular concentrations				Ratio K ÷ Na	Gram moles × 10^3 = moles per liter × volume			
					K	Na	K + Na	Halide		K	Na	K + Na	Halide
	days	*ml.*	*ml.*										
8.8 (High)	0	11.56 11.23	11.40	5.75	0.5211	0.1101	0.6312	0.6182	4.72	5.940	1.255	7.195	7.047
	5	13.00 12.65	12.83	5.75	0.5035	0.1231	0.6266	0.6164	4.09	6.460	1.579	8.039	7.908
	10	13.66 13.27	13.47	5.90	0.5163	0.1091	0.6254	0.6133	4.73	6.956	1.469	8.425	8.261
8.2 (Control)	0	11.18 11.10	11.40	5.75	0.5211	0.1101	0.6312	0.6182	4.72	5.940	1.255	7.195	7.047
	5	12.11 11.83	12.25	5.65	0.5101	0.1162	0.6263	0.6158	4.34	6.254	1.424	7.678	7.544
	10	12.62 12.43	12.82	5.70	0.4946	0.1258	0.6204	0.6121	3.93	6.341	1.613	7.954	7.847

* In order to establish the reproducibility of the rate of growth, volume measurements were made on two sets of cells at each pH. The results indicate that the growth curves are reasonably reproducible.

† These values were obtained as follows: The two volume measurements were averaged and in the case of the control the averaged values were multiplied by the factor 11.40 ÷ 11.14.

TABLE III

Gain in Moles of Potassium in Section A, Experiment 1

Time	Gain at pH 8.8	Gain at pH 8.2	(Gain at pH 8.8) ÷ (Gain at pH 8.2)		
			Observed	Calculated	
				Entering as KOH	Ionic exchange
days					
0– 3	0.227	0.083	2.74	5.72	1.44
3–10*	0.240	0.087	2.76	5.44	0.85
10–15	0.074	0.015	5.0	5.43	0.98
			Av. = 3.5	Av. = 5.53	Av. = 1.09

* No calculation is made for the period between the 3rd and the 6th day since the table shows a loss in moles at pH 8.2 (this is probably due to experimental error).

would be expected if potassium entered as KOH at the pH values stated. In this connection we may consider the following:

(*a*) In spite of the flow of sea water, the pH just outside the protoplasm[10] undoubtedly rose greatly as the result of photosynthesis and this rise was much greater in the controls than in the cells placed in sea water at 8.8. Hence the difference between the two lots of cells was undoubtedly very much less than that assumed in making the calculation. This will be discussed presently (p. 740).

(*b*) The high pH may change the protoplasm (*e.g.* by leaching as in the case of *Nitella*[11]). Such disturbing factors are probably responsible for the fact that in general growth does not go on increasing indefinitely as the pH is raised, but reaches an optimum and then declines.

In the absence of these disturbing factors we might expect the behavior of potassium to proceed more nearly according to calculation and this seems to be the case. When, for example, we raise the concentration of KCl in the sea water from 0.011 M to 0.024 M (without changing the pH) a calculation of data in a former paper[3] shows that the rate of entrance should increase 2.95 times (calculation on the basis of ionic exchange gives about the same). In 20 days the observed increase was 2.24 times. The discrepancy is not great and may be due to small differences in pH.

B. At Low pH: Flowing Sea Water

In these experiments the cells were exposed to running sea water (day and night) at pH 6.8 and at pH 8.2. They were kept out of doors (shaded from direct sunlight). In the first experiment the pH was adjusted in the manner previously described and the fluctuations during the experiment did not exceed 0.2 pH unit. The results are given in Table IV.

Here the rate of growth was greater in the control than at the low pH, and the rate of entrance of moles of potassium was from 2.2 to 4 times as great. This is a smaller difference than we should expect if there were no photosynthesis. Indeed we should expect potassium to come out at the low pH. However, it has been pointed out (p. 734) that the photosynthetic effect makes it impossible to make reliable quantitative calculations.[12]

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[10] *I.e.*, in the cell wall and in the film between the protoplasm and the cell wall where the flow of sea water would have relatively little effect.

[11] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 99. Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, **35**, 988.

Another variation is illustrated by Experiment 2. This was actually a preliminary experiment, but it raises an interesting point. Here the behavior of cells exposed to low pH and normal sea water was about the same (Table V). This is not surprising in view of the special conditions (this is discussed on p. 740).

TABLE IV

Analytical Data on Saps of Valonia

Section B, Experiment 1 (pH Relatively Constant)

Nominal pH of sea water	Time	pH of sap	Molecular concentrations				Ratio K ÷ Na	Volume*	Gram moles × 10³ = moles per liter × volume			
			K	Na	K + Na	Halide			K	Na	K + Na	Halide
	days							*ml.*				
8.2 (Control)	0	5.90	0.4782	0.1634	0.6416	0.6180	2.93	7.30	3.491	1.193	4.684	4.511
	3	5.90	0.4673	0.1668	0.6341	0.6103	2.80	7.85	3 668	1.310	4.978	4.791
	6	5.85	0.4835	0.1467	0.6302	0.6232	3.29	8.17	3.950	1.198	5.149	5.091
	10	5.70	0.4790	0.1470	0.6260	0.6128	3.26	8.29	3.972	1.219	5.191	5.080
	15	5.65	0.4727	0.1528	0.6255	0.6128	3.09	8.49	4.013	1.297	5.310	5.203
6.8 (Low)	0	5.90	0.4782	0.1634	0.6416	0.6180	2.95	7 30	3.491	1.193	4.684	4.511
	3	5.75	0.4703	0.1624	0.6327	0.6122	2.89	7.52	3.536	1.221	4.757	4.602
	6	5.70	0.4677	0.1649	0.6326	0.6201	2.83	7.71	3.606	1.270	4.876	4.781
	10	5.75	0.4638	0.1651	0.6289	0.6111	2.81	7.81	3.622	1.290	4.912	4.773
	15	5.65	0.4661	0.1637	0.6298	0.6182	2.85	7.99	3.724	1.308	5.032	4.909

* See note (*) under Table II. The factor to bring both volumes to the same value at the start was 7.30 ÷ 7.07 applied to the lot at pH 6.8.

The cells were exposed in the same way as in Section A, but the adjustment of the pH to 6.8 was accomplished by shaking with 0.6 N HCl without aeration. Here the pH fluctuated as stated below.

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[12] To calculate the pH at which potassium should begin to come out involves an accurate knowledge of individual ion activities which is not possible to obtain. *Cf.* Guggenheim, E. A., *J. Phys. Chem.*, 1929, **33**, 842; MacInnes, D. A., The meaning and calibration of the pH scale, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1933, **1**, 190.

The pH was determined at 24 hour intervals and each time it was found that an increase had taken place and more acid had to be added to bring it back to the neighborhood of 6.8. The highest point reached was 7.4 and as the experiment proceeded the magnitude of the rise decreased. The explanation seems to be that when sea water is acidified it tends to remain supersaturated with respect to free CO_2 for a period which depends largely on the amount of agitation it receives.[13]

The observed rise of the pH to 7.4 is not, of course, sufficient to make the value of (K_o) (OH_o) for the low pH group equal to that for the normal group but there is good reason to believe that (OH_i) for the low group may have been less than usual. For it is well known that free CO_2 in the sea water penetrates into the vacuole very rapidly and produces a marked decrease in the pH. Hence in sea water supersaturated with CO_2 the internal pH ought to be lower. The pH measurements on the sap do not show this, but these measurements were made on saps extracted in the morning when the sea water pH had risen. However, the following supplementary experiments described below indicate that the internal pH actually can be changed to a considerable extent by rapidly changing the outside pH under these conditions.

TABLE V

Analytical Data on Saps of Valonia

Section B, Experiment 2 (pH Fluctuating)

Nominal pH of sea water	Time	pH of sap	Molecular concentrations				Ratio K ÷ Na	Volume	Gram moles × 10^3 = moles per liter × volume			
			K	Na	K + Na	Halide			K	Na	K + Na	Halide
	days							*ml.*				
6.8* (Low)	0	6.15	0.4952	0.1408	0.6360	0.6275	3.52	8.73	4.323	1.229	5.552	5.478
	3	6.15	0.4929	0.1408	0.6337	0.6325	3.50	9.04	4.456	1.273	5.729	5.689
	6	6.00	0.4877	0.1381	0.6258	0.6269	3.53	9.15	4.462	1.264	5.727	5.736
	10	5.90	0.4870	0.1388	0.6258	0.6250	3.51	9.27	4.515	1.287	5.802	5.794
	15	6.00	0.4811	0.1470	0.6281	0.6244	3.27	9.39	4.518	1.380	5.898	5.863

* For corresponding control experiment at pH 8.2 see Table I.

About 100 cells, whose sap had an average pH of 5.9, were placed in 500 cc. of sea water the pH of which had been reduced by the addition of 0.6 N HCl to

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[13] We find that if all the HCl required to bring the pH of 16 liters of sea water down to the neighborhood of 7 is added at once, it requires about 5 hours of aeration, at a rate roughly estimated to be about 10 liters of air a minute, to relieve the supersaturation.

TABLE VI

Analytical Data on Saps of Valonia

Section C, Experiment 1 (Cells in Bottles)

Nominal pH of sea water	Time	Volume*	Adjusted volume†	pH of sap	Molecular concentrations				Ratio K ÷ Na	Gram moles × 10^3 = moles per liter × volume			
					K	Na	K + Na	Halide		K	Na	K + Na	Halide
	days	*ml*	*ml.*										
8.8 (High)	0	10.48 10.90	10.69	5.95	0.5195	0.1104	0.6299		4.71	5.553	1.180	6.733	
	2	11.17 11.75	11.46	5.75	0.5173	0.1135	0.6308	0.6139	4.56	5.928	1.301	7.229	7.035
	6	11.58 11.98	11.78	5.80	0.5189	0.1097	0.6286	0.6182	4.73	6.113	1.292	7.405	7.282
	9	11.74 12.12	11.93	5.75	0.5263	0.1019	0.6282	0.6254	5.16	6.278	1.216	7.494	7.461
8.2 (Control)	0	10.07 9.60	10.69	5.95	0.5195	0.1104	0.6299		4.71	5.553	1.180	6.733	
	2	10.70 10.26	11.38	5.70	0.5201	0.1091	0.6292	0.6196	4.77	5.919	1.242	7.161	7.051
	6	11.20 10.36	11.71	5.75	0.5233	0.1070	0.6303	0.6260	4.89	6.128	1.253	7.381	7.330
	9	11.33 10.60	11.92	5.75	0.5145	0.1256	0.6403	0.6302	4.09	6.133	1.498	7.631	7.511
6.8 (Low)	0	10.27	10.69	5.95	0.5195	0.1104	0.6299		4.71	5.553	1.180	6.733	
	2	10.60	11.03	5.65	0.5084	0.1156	0.6240	0.6196	4.40	5.608	1.275	6.883	6.834
	6	10.64	11.08	5.75	0.5026	0.1193	0.6220		4.21	5.568	1.322	6.890	
	9	10.71	11.15	5.70	0.4964	0.1289	0.6253	0.6128	3.85	5.534	1.437	6.971	6.832

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* See note (*) under Table II.

† See note under Table I. Factors for bringing the volumes to the same value at the start are 10.69 ÷ 9.84 applied to the control and 10.69 ÷ 10.27 applied to the lot at pH 6.8.

6.5 (without aeration). 15 minutes later the average pH of the cell sap was 5.6, and 30 minutes later it was 5.0. The sea water was then shaken vigorously to remove the excess CO_2, and after 15 minutes' exposure the pH of the sap had risen to 5.85.

From this experiment it may be deduced that in the low pH group of Experiment 2 the inside pH was for a considerable part of the time less than normal so that the value of $(K_o)(OH_o) - (K_i)(OH_i)$ was greater than usual. It is useless to attempt to deal with the question quantitatively, but it may be said with confidence that the gradients of the low and normal groups of the experiment must have been much closer than the pH values given in Table V would indicate.

TABLE VII

*Analytical Data on Saps of Valonia**

Section C, Experiment 2 (Cells in Tubes)

Nominal pH of sea water	Time	pH of sap	Molecular concentrations			Ratio K ÷ Na	Volume†	Gram moles × 10^3 = moles per liter × volume		
			K	Na	K + Na			K	Na	K + Na
	days						*ml.*			
8.8 (High)	0	6.15	0.5051	0.1234	0.6285	4.09	13.29	6.713	1.640	8.353
	2	5.90	0.5017	0.1272	0.6289	3.95	13.63	6.838	1.734	8.572
	5	5.90	0.5059	0.1173	0.6232	4.31	13.92	7.042	1.632	8.674
	10	6.00	0.4857	0.1415	0.6272	3.43	14.19	6.892	2.008	8.900
8.2 (Control)	0	6.15	0.5051	0.1234	0.6285	4.09	13.29	6.713	1.640	8.353
	2	5.90	0.5011	0.1272	0.6283	3.94	13.66	6.846	1.736	8.582
	5	5.90	0.5030	0.1258	0.6288	4.00	14.01	7.048	1.762	8.810
	10	5.95	0.4879	0.1395	0.6274	3.50	14.22	6.938	1.984	8.922
6.8 (Low)	0	6.15	0.5051	0.1234	0.6285	4.09	13.29	6.713	1.640	8.353
	2	5.90	0.5026	0.1197	0.6223	4.20	13.35	6.710	1.598	8.308
	5	6.00	0.4834	0.1313	0.6147	3.69	13.61	6.579	1.787	8.366
	10	5.75	0.4805	0.1367	0.6172	3.51	13.77	6.616	1.882	8.498

* Owing to lack of material no determination of halide was made.

† See note under Table I. The factors for bringing all volumes to the same value at the start are 13.29 ÷ 12.54 applied to the control and 13.29 ÷ 13.27 applied to the lot at pH 6.8.

C. At High and Low pH: Sea Water Not Flowing

In these experiments no effort was made to prevent the change of pH due to the photosynthetic removal of CO_2. On the contrary, the effect was exaggerated by illuminating the cells constantly with artificial light. In each experiment in this section there were three groups of cells. One was exposed to sea water at pH 6.8, one at pH

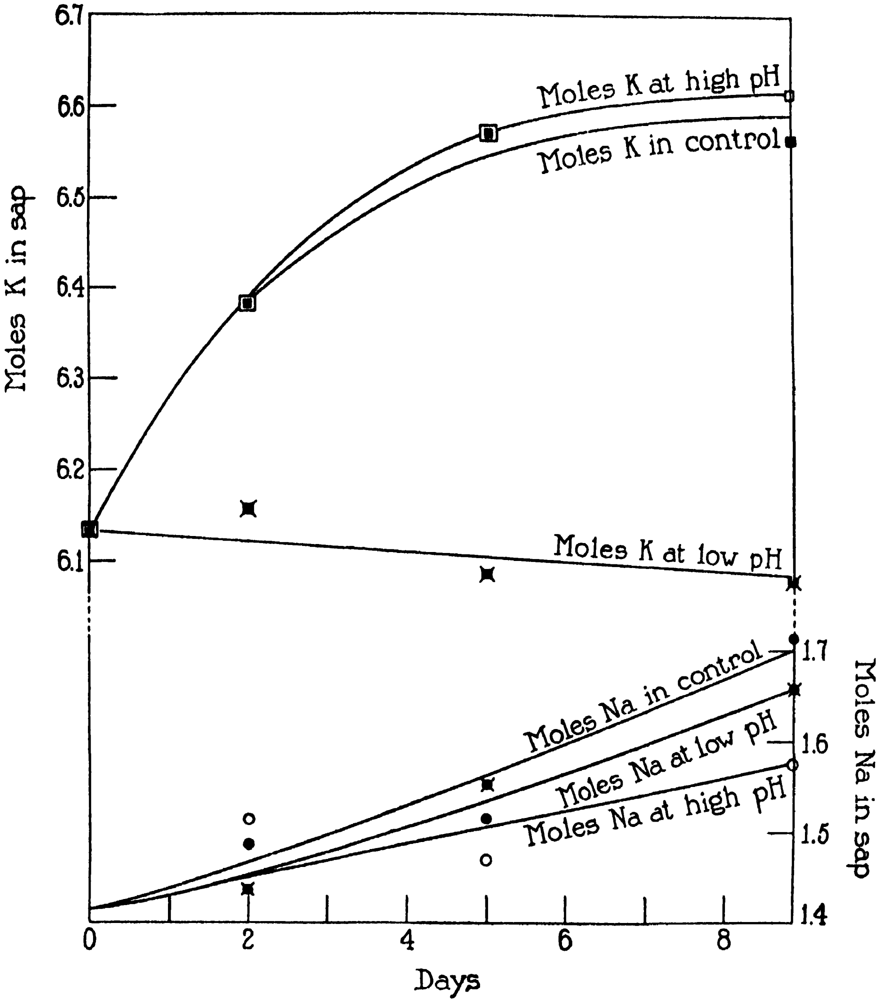

FIG. 2. Moles of potassium and of sodium entering *Valonia* at high pH (about 8.8) and in a control experiment where the pH just outside the protoplasm was about 8.2 at the start and probably rose to the neighborhood of 8.8 during the experiment as the result of photosynthesis. (Sea water not flowing.) The curves labelled "low pH" relate to an experiment in which the pH was 6.8 at the start and probably did not rise much since the supply of CO_2 was reduced. Average of Experiments 1 and 2, Section C. The curves are drawn free-hand to give an approximate fit.

8.8, and one at 8.2. These were the pH values at which the experiments began. Rapid and extensive changes due to the photosynthesis of the cells took place. These will be discussed presently.

The results are given in Tables VI and VII (see also Fig. 2[14]).

In Experiment 1 the cells were exposed in bottles holding 250 cc., the light being furnished by a 500 watt light at a distance of about 18 inches. About 2 inches of distilled water was interposed between cells and light to serve as a heat filter.

In Experiment 2 the cells were exposed in large glass tubes the light being furnished by nine 100 watt lights in three rows: the tubes containing the cells were placed 18 inches above the lights. As before, about 2 inches of distilled water was interposed as a heat filter.

The sea water was changed daily. This change reduced the pH temporarily but it soon rose again under the influence of photosynthesis.

It is evident that at both the high and the normal pH the rate of growth and the rate of increase in moles of potassium was approximately the same. This is to be expected in view of the effects of photosynthesis, which may be summarized as follows:

It is well known that marine plants increase the pH of the sea water during the day time. Thus McClendon[15] reports that in the Tortugas the pH of the sea water over well lighted bottoms rich in vegetation rises from 8.18 at night to 8.35 during the day. Crozier[16] states that in Fairyland Creek, a tidal inlet in Bermuda, where *Valonia* formerly grew luxuriantly, the pH at the head of the creek rises to 8.3 during the day, while in the Great Sound at the north it is 8.07. He considers this to be due to photosynthetic abstraction of CO_2. The same investigator also found that *Valonia* kept in an aquarium raises the pH of normal sea water to the neighborhood of 9.5, by removing the CO_2 for photosynthesis.

It was therefore to be expected that in the limited volume of sea water here employed the pH would rise rapidly (as found, for example, by Osterhout and Haas in experiments on *Ulva*[17]).

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[14] This is based on an average of Experiments 1 and 2. Since the times were not in all cases the same in both experiments some of the values were estimated by graphic interpolation which in this case involved little or no error.

[15] McClendon, J. F., *Carnegie Institution of Washington, Pub. No. 252*, 1918, 254.

[16] Crozier, W. J., *J. Gen. Physiol.*, 1918–19, **1**, 581.

[17] Osterhout, W. J. V., and Haas, A. R. C., *Science*, 1918, **47**, 420; *J. Gen. Physiol.*, 1918–19, **1**, 1.

In one experiment, for example, we found that when 2 liters of sea water were exposed to about 600 cells (of about 200 cc. volume) the pH rose from 8.3 to 8.8 in 5 hours and to 9.4 overnight (in this case continuous artificial illumination was used).

In another experiment with natural illumination (outdoors in the shade) the pH of 100 cc. of sea water in contact with 30 cells (with a volume of about 15 cc.) rose from 8.3 to 9.3 in 10 hours.

In neither case was the sea water stirred so that it seems probable that in the vicinity of the cells the change in the pH was much more rapid. Doubtless the pH of the sea water in contact with the outer layer of the protoplasm becomes greater than normal as soon as the cell is illuminated.

In other experiments sea water at pH 9.0 was exposed to cells and illuminated. Here also there was a rapid increase in the pH but the final value was only 9.3 to 9.4. Thus it appears that there is a definite limit to which the pH of Bermuda sea water can be raised by the removal of CO_2, and that this limit is about the same whether the sea water before the experiment is at normal or high pH. It was also found, as stated previously, that when the pH rose to 9.4 $CaCO_3$ was precipitated. A study of the dissociation curve of CO_2 in sea water[18] shows that at pH 9.4 an appreciable part of the total amount of CO_2 in the system must be in the form of carbonate ion. But the sea water where the bottom is entirely calcareous is certainly saturated or nearly saturated with respect to $CaCO_3$,[19] so that when the activity of CO_3 ion is raised $CaCO_3$ is salted out. Hence the pH is buffered at that point.[20]

It was further observed that the pH of sea water raised by photosynthesis could be lowered readily to its original value by equilibrating it again with the CO_2 of the atmosphere by aeration.[21]

It should be pointed out that CO_2 from two sources will tend to offset the loss of CO_2 in the sea water imbibed in the cellulose wall: CO_2 may diffuse in from the surrounding sea water and out from the protoplasm (where it is produced during respiration). Respiration is, of course, going on constantly but obviously the amount of CO_2 produced is less than the loss due to photosynthesis as long as the cell is illuminated. When illumination ceases it becomes important as the following experiment shows.

A group of cells in sea water in a closed bottle were placed outside in the shade so that in 10 hours the pH of the sea water rose from 8.3 to 9.3. They were then kept in the dark for 12 hours and the pH fell to 8.6. They were again illuminated and the pH rose to 9.3. Finally they were darkened again for 12 hours and the pH fell to 7.9.[22] In a control experiment in which sea water without cells was treated in exactly the same way the pH (which was 8.3) did not change.

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[18] Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, **9**, 255.

[19] McClendon, J. F., *Carnegie Institution of Washington*, *Pub. No. 252*, 1918, 256.

[20] Part of the buffer capacity of sea water is due to other acids than carbonic, for example, boric, phosphoric, silicic, and possibly arsenic, but the amount of any of these is so small that they may be neglected in this discussion.

[21] This, of course, refers to sea waters which had not been allowed to remain long enough at the high pH to lose much calcium carbonate.

Such effects of photosynthesis appear to be chiefly responsible for the difference between Sections A and C.

In the latter these effects were allowed to develop fully. The illumination was continuous and the sea water over the cells was changed just enough to prevent the possible exhaustion of other substances in the sea water which the cells might need. Under these conditions we may expect that in the control and high pH sea water the actual pH would be most of the time near 9.4. Daily determinations of the pH showed this to be the case, and when the sea waters were replaced by fresh samples having the correct pH it rose in a few hours to 9.4. The pH just outside the protoplasm would of course rise more rapidly than in the main body of the sea water. It is therefore not surprising that the normal and high pH groups in Section C behaved in the same way.

In Section A the pH was controlled as far as possible by passing the sea water fairly rapidly over the cells. The CO_2 content was restored by frequent aeration. The illumination was natural and therefore intermittent, so that for part of the time photosynthesis was abolished. The measured pH of the sea water did not change much in either group. Here, as would be expected, there was a distinct difference between the control and the high pH groups.

Let us now consider the behavior of cells at low pH. In both experiments the rate of growth was much less at the low pH than at either normal or high pH. Potassium moles did not change much. It is important to note that although in these low pH groups the external pH was much higher than the nominal 6.8 because of photosynthesis, it could not rise to the point reached by the normal and high pH groups. This is because in the process of reducing the sea water pH about 19/20 of the bicarbonate originally present was removed.

In one experiment for example in which about 600 cells (of about 200 cc. volume) were exposed with constant artificial illumination to 2 liters of sea water originally at 6.8 the pH rose to 7.7 in 5 hours and overnight to 8.4, but not higher after further exposure. It seems probable therefore that even in the layer of sea water just outside the protoplasm the pH did not rise much above 8.4. This would explain the difference between the cells in low pH as contrasted with those at normal and high pH.

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[22] This indicates that the sea water was slightly supersaturated with CO_2, which might easily happen in a small volume of sea water in a small tightly stoppered bottle.

Summarizing the experiments with illumination, constant or intermittent, we find that in all cases where a pH difference was maintained the growth and rate of entrance of potassium were greater at the higher pH.

In those cases where, in spite of nominal differences of pH, the cells behaved alike it is probable that, owing to photosynthesis, or supersaturation of the sea water by CO_2, little or no actual difference existed.

D. Experiments in the Dark

In an effort to avoid the changes in the pH of the sea water due to photosynthesis, some experiments were carried out in the dark at high (8.9) and low (6.9) pH with a control at pH 8.2. In these experiments the cells were exposed either in 2 liter bottles or large Pyrex tubes darkened by the application of several coats of black Duco. A rapid flow of sea water was maintained in all cases. In normal and high sea water in contact with cells there was a tendency for the pH to fall slightly but this was easily corrected by aeration. In the second experiment of this series the potassium content of the sea water was raised to five times the normal value.[23]

The results of the experiments in the dark may be summarized as follows. There was no definite evidence of growth at any of the pH values studied, even when the gradient was raised by increasing the potassium content of the sea water to five times normal. Nor was there any decisive drift in the rate of entrance of potassium moles except that in the low pH group there is some indication that potassium came out of the cells, especially in the experiment in which the concentration of the potassium in the sea water was normal.

The fact that the uptake of electrolytes depends on light accords with the experience of Hoagland[24] and his collaborators, and of M. M. Brooks,[25] and with our own earlier experiments.[26]

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[23] This was done as described in a previous paper by the addition to normal sea water of suitable amounts of artificial sea water, in which all the sodium was replaced by potassium. *Cf.* footnote 3.

To explain the failure of cells to grow and take in potassium we may consider the following:

In the first place account must be taken of the effect of respiration in the dark. The CO_2 produced in the cell may diffuse out and produce a pH change in the layer of sea water next to the outside of the protoplasm. The following experiments show that changes do occur but they are not nearly as extensive as those produced by photosynthesis.[27]

In these experiments sea waters at three pH's after adequate aeration were exposed to cells in darkened Pyrex bottles and the changes compared with those in the same sea waters not exposed to cells. The results are given in Table VIII.

The pH tends to fall slightly, not enough to explain the failure of the cells to grow in the dark at high pH and take in potassium when its concentration outside was five times normal. We must bear in mind, however, that owing to respiration the pH just outside the protoplasm may be considerably lower than in the main body of sea water.

TABLE VIII

pH of Sea Water Surrounding the Cells

Time	Low pH		Control		High pH	
	Control	Experiment	Control	Experiment	Control	Experiment
hrs.						
0	6.8	6.8	7.9	7.9	8.6	8.5
24	6.9	6.8	7.9	7.7	8.5	8.3
96	7.0	6.7	8.1	7.9	8.4	8.1

It seems much more probable that the failure to grow is due to the inability of the cell to manufacture cellulose in the dark. This might prevent the stretching of the cell wall and so render growth impossible. This is the more likely since there is little or no storage of carbohydrate in these cells.

It might be supposed that even in the absence of growth potassium and sodium would continue to enter. If this occurred the osmotic pressure would rise and put an increasing pressure on the cell wall and this might be expected to check the further entrance of electrolytes. It would seem that this situation needs to be clarified by further investigation since in *Nitella* the concentration of electrolytes and the osmotic activity in the sap rise far above those in the pond water, but it must be remembered that in the latter they are exceedingly small and that in the sap they never reach the values found in *Valonia.*

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[24] Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1923–24, **6**, 47. Hoagland, D. R., Hibbard, P. L., and Davis, A. R., *J. Gen. Physiol.*, 1926–27, **10**, 121.

[25] Brooks, M. M., *Protoplasma*, 1926, **1**, 305.

[26] Cooper, W. C., Jr., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 117.

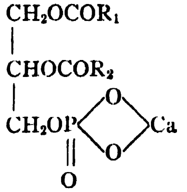
[27] This, of course, is not unexpected for respiration is going on steadily even when the cell is illuminated and therefore CO_2 is coming out of the vacuole, but clearly so slowly that it does little to offset the photosynthetic abstraction of CO_2.

In experiments in the dark at pH 6.8 (K_o) (OH_o) is less than (K_i) (OH_i) and we should therefore expect potassium to come out of the cell, as indeed it did. In 15 days the loss in K moles was 4.4 per cent as compared with a loss of 0.12 per cent in the control and a gain of 1.9 per cent at pH 8.8. The rate of decrease of potassium moles was rather slow and it was compensated by a gain of sodium moles. This would be expected since (Na_o) (OH_o) is greater than (Na_i) (OH_i).

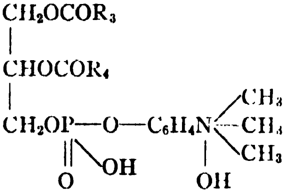
The third possible explanation of the failure of the cells to grow in the dark and take in potassium derives from the suggestion[28] that the entrance of electrolytes may depend on the presence in the protoplasm of certain acids which act as carriers of potassium (see p. 747), as guaiacol acts in a model which accumulates potassium. If such carriers exist in *Valonia* they may be fugitive compounds present only during photosynthesis.

In view of the fact that we are inclined to emphasize the importance of a carrier in cell permeability, the following may be of some significance. Hundeshagen[29] has synthesized a diglyceryl stearyl phosphoric acid, the sodium and potassium salts of which are more soluble in non-polar organic solvents than in water: this applies in even greater degree to the calcium salt. Recently Chibnall and Channon[30] have demonstrated the presence of a very similar compound in the form of the Ca salt in a variety of cabbage (*Brassica oleracea*, L.). Still more recently Smith and Chibnall[31] have extracted a similar compound from *Dactylis glomerata*, L. It is interesting, therefore, to consider whether the carrier in the protoplasm may not be some such compound.

It may be noted that the compounds described are related to the phosphatides according to the following scheme[32]



The Ca salt of the diglyceryl phosphoric acid



A phosphatide

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[28] Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931–32, **15**, 667.

[29] Hundeshagen, F., *J. prakt. Chem.*, 1883, **28**, N. S. **2**, 219.

[30] Chibnall, A. C., and Channon, H. J., *Biochem. J.*, London, 1927, **21**, 225.

[31] Smith, J. A. B., and Chibnall, A. C., *Biochem. J.*, London, 1932, **26**, 1342.

[32] Here R_1, R_2, etc., are different fatty acid residues (each taken once).

This is of interest in connection with the claims[33] of Hansteen-Cranner, Grafe, Magistris, and others that phosphatides are present in the protoplasmic surfaces and play an important part in determining their permeability.

If the substances concerned resembled those described by Hundeshagen we might expect them to collect in the non-aqueous protoplasmic surfaces and to be very slowly dissolved out by distilled water: this action would be checked by the addition of calcium to the distilled water. To this extent the picture fits the situation found in the protoplasmic surface of *Nitella.*[34] Whether HCl and NaOH would dissolve these salts (when mixed with other substances) more rapidly than distilled water (as we might infer from the experiments on *Nitella*) is not known but may not be impossible.

There are, however, other changes which accompany the pH changes of the sea water, which may have some effect. Thus the changes in the CO_2-HCO_3-CO_3 buffer system may be important. For example, at both high and low pH in our experiments the concentration of the bicarbonate ion was reduced. Hence, if, as M. M. Brooks[35] believes, sodium or potassium bicarbonate can pass through the protoplasm as such, account must be taken of this factor. But both experiment[36] and theory seem opposed to this.[37] Further it might be thought that the reduction of bicarbonate outside would change the rate of exchange of bicarbonate ion for chloride ion. These points, however, cannot be settled without further experiment.

It may be added that the temperature of illuminated cells is higher than that of the surrounding solution.

DISCUSSION

The effect of pH might be thought to indicate that potassium penetrates as K^+ which enters in exchange for H^+ produced in the cell

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[33] Hansteen-Cranner, B., Zur Biochemie und Physiologie der Grenzschichten lebender Pflanzenzellen, Christiania, Grøndahl and Søns, 1922. (Meldinger fra Norges Landbrukshøiskole, 1922, **2,** Nos. 1 and 2.) Grafe, V., *Biochem. Z.*, Berlin, 1925, **159,** 445; 1929, **205,** 256; *Beitr. Biol. Pflanz.*, 1928, **16,** 129. Grafe, V., and Horvat, V., *Biochem. Z.*, Berlin, 1925, **159,** 449. Grafe, V., and Magistris, H., *Biochem. Z.*, Berlin, 1925, **162,** 366; 1926, **176,** 266; **177,** 16. Grafe, V., and Ose, K., *Biochem. Z.*, Berlin, 1927, **187,** 102. Grafe, V., and Freund, K., *Beitr. Biol. Pflanz.*, 1928, **16,** 140. Magistris, H., *Biochem. Z.*, Berlin, 1929, **210,** 85. Magistris, H., and Schäfer, P., *Biochem. Z.*, Berlin, 1929, **214,** 440. Thierfelder, H., and Klenk, E., Die Chemie der Cerebroside und Phosphatide, Berlin, Julius Springer, 1930.

[34] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17,** 87.

[35] Brooks, M. M., *Pub. Health Rep., U. S. P. H. S.*, 1923, **38,** 1470. These results might be explained equally well as due to the entrance of KOH.

[36] Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13,** 695.

[37] Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, **35,** 984, 993, 1002.

(the ions passing as such through the protoplasmic surface). But the calculations of Table III show that at the pH values stated the change in the rate of entrance would then be much less than actually observed. These calculations are made as follows.

The rate of entrance would be proportional to (K_o) (H_i) – (K_i) (H_o). Hence,[38] other things being equal, we may put for the first period in Table I

$$\frac{\text{Rate at pH 8.8}}{\text{Rate at pH 8.2}} = \frac{0.12\ (10^{-6.15}) - 0.4952\ (10^{-8.8})}{0.12\ (10^{-6.15}) - 0.4952\ (10^{-8.2})} = 1.44$$

In this way we obtain the values in Table III. The average for the three periods is 1.09 which does not agree so well with the average observed value of 3.5 as does that of 5.53 calculated on the assumption that potassium enters as KOH. The effects of photosynthesis would make the observed value less than the calculated (see p. 740). But there seems to be no way of explaining the discrepancy between the values of 1.09 and 3.5 except by assuming that ionic exchange is unimportant.

As ionic exchange has recently been discussed elsewhere[37] it is not necessary to deal with it at length here. We need only say that in general its rôle appears to be a very subordinate one in the entrance of electrolytes.

Let us now consider in more detail the idea that potassium enters chiefly as KOH. If this should come about as in certain models[28] (where potassium accumulates) we may suppose that KOH unites with an acid H*X* in the protoplasmic surface to form K*X* which reacts with CO_2 in the sap to form $KHCO_3$. The rate of entrance would then be proportional[39] to (K_o) (OH_o) – (K_i) (OH_i) and must therefore increase as the acidity of sap increases so that we may expect that as respiration increases the rate of entrance of electrolytes and of growth will also increase. This seems to be true in general since young, rapidly growing cells usually have a relatively high rate of respiration. This has been discussed elsewhere.[37, 40]

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[38] Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 277.

[39] Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529. Osterhout, W. J. V., Kamerling, S. E., and Stanley, W. M., *J. Gen. Physiol.*, 1933–34, **17**, 445, 469.

[40] We find that in models (footnote 28) when CO_2 stops bubbling the growth falls off.

The function of respiration in this connection appears to be twofold, not only keeping (OH_i) low but also furnishing HCO_3 for exchange with Cl.[41] An exchange of this sort is assumed in order to account for the fact that potassium accumulates as KCl.[37] It might also be suggested that HCl enters to displace CO_2 which can readily escape.

The product (K_i) (Cl_i) is greater than (K_o) (Cl_o) and it is evident that energy is required to bring this about. Doubtless this is derived from respiration but the mechanism requires elucidation. In models[28, 37, 39] we are able to raise the product (K_i) (HCO_{3_i}) far above (K_o) (HCO_{3_o}) by simply bubbling CO_2 inside; *i.e.*, without using any of the energy derived from the formation of CO_2, so that we bring about accumulation by using what is ordinarily regarded as a waste product of the cell.

When the rate of entrance of KOH increases slowly there may be little or no rise in the pH of the sap[42] because it may be prevented by the exchange of HCO_3^- for Cl^- [41] or by the production of acid in the cell: but when KOH enters rapidly there may be a local rise in pH just inside which will check the entrance of KOH and this may explain why the rate of entrance at high external pH is less than the calculation would indicate: a rise in pH in the sap as a whole is found in some cases (Tables I and II). We expect little or no change in the concentration of potassium or of halide since water will enter because of the increased osmotic pressure in the sap (this agrees with experiments): in consequence growth increases (Tables I and II).

The opposite picture is seen when the external pH is lowered and the entrance of potassium falls off (Fig. 2, Tables IV to VII).

Since the rate of entrance depends on the external pH it seems possible that there may be considerable difference between light and darkness. In the light the value of (OH_o) just outside the protoplasm would increase as the result of photosynthesis, thus favoring the entrance of KOH.[43] In darkness the value of (H_o) just outside the protoplasm would increase as the result of respiration, thus favoring the entrance of HCl.

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[41] Regarding evidence for this see footnote 37. We assume that HCO_3 and Cl pass in molecular form through the protoplasmic surface.

[42] If KOH react with HX in the protoplasm to form KX and this in turn with H_2CO_3 to form $KHCO_3$ in the sap the end result is the same as if KOH penetrated as such (which would increase the pH of the sap).

In conclusion it may be desirable to point out that the effects of photosynthesis here described would not alter the conclusions of former papers in cases where variation in the external pH is not significant, as for example in comparing the entrance of potassium from sea water containing 0.011 M and 0.24 M potassium.[2] But in dealing with the exit of potassium the external pH is important and the calculations[44] would be misleading when based on the measured pH of the sea water making no allowance for photosynthesis (unless compensated by a rise in the pH of the layer of sap adjoining the protoplasm).

Such considerations emphasize the importance of a constant flow of sea water to control the pH and other variables as far as possible.

Let us now consider the relation between sodium and potassium. Calculations similar to that given on p. 731 show that raising the external pH should not increase the entrance of sodium so much as that of potassium. This agrees qualitatively with the data (Tables I and II) which show that the value of K ÷ Na is greater at pH 8.8 than at pH 8.15.[45] Possibly this is due in part to changes in the protoplasm caused by the higher pH.[46]

The fact that in this case the penetration of electrolytes depends on the external pH leads us to ask whether this is of general application.[47] Since as a rule growth depends on pH the following interpretation is offered for consideration. As the pH of the external solution rises from the minimum at which growth is possible the penetration of electrolytes increases, thereby raising the internal osmotic pressure and causing absorption of water, as seen in models.[28] When the pH rises above the optimum secondary changes occur; *e.g.*, such alterations of the protoplasm as are seen in experiments on *Nitella*.[48]

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[43] If this went far enough it might affect the pH of the sap (as when NH_3 enters) unless compensated by the production of CO_2 in the sap. But since the volume of sap is so large it is not probable that the effect could ordinarily be detected.

[44] Such calculations cannot have much accuracy since we do not know the individual ion activities. *Cf.* footnote 12.

[45] The decline of this ratio in the control might raise the question of injury but the cells appeared normal and the growth was good.

[46] *Cf.* Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, **35**, 1017.

[47] Regarding entrance of cations as hydrates, see Osterhout, W. J. V., *Science*, 1912, **36**, 571; *Ergebn. Physiol.*, 1933, **35**, 971, 973, Bibliography references 119, 193*a*, 194, 195.

SUMMARY

It would be natural to suppose that potassium enters *Valonia* as KCl since it appears in this form in the sap. We find, however, that on this basis we cannot predict the behavior of potassium in any respect. But we can readily do so if we assume that it penetrates chiefly as KOH. We may then say that under normal conditions potassium enters the cell because the ionic activity product (K) (OH) is greater outside than inside. This hypothesis leads to the following predictions:

1. When the product (K) (OH) becomes greater inside (because the inside concentration of OH^- rises, or the outside concentration of K^+ or of OH^- falls) potassium should leave the cell, though sodium continues to enter. Previous experiments, and those in this paper, indicate that this is the case.
2. Increasing the pH value of the sea water should increase the rate of entrance of potassium, and *vice versa*. This appears to be shown by the results described in the present paper.

It appears that photosynthesis increases the rate of entrance of potassium by increasing the pH value just outside the protoplasm. In darkness there is little or no growth or absorption of electrolytes.

The entrance of potassium by ionic exchange (K^+ exchanged for H^+ produced in the cell), the ions passing as such through the protoplasmic surface, does not seem to be important.

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[48] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 99. Below the minimum and above the maximum pH visible alterations in the protoplasm occur and in time there is permanent injury or death.

THE PHOTOTROPIC EFFECT OF POLARIZED LIGHT

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PLATE 1

(Accepted for publication, January 31, 1934)

I

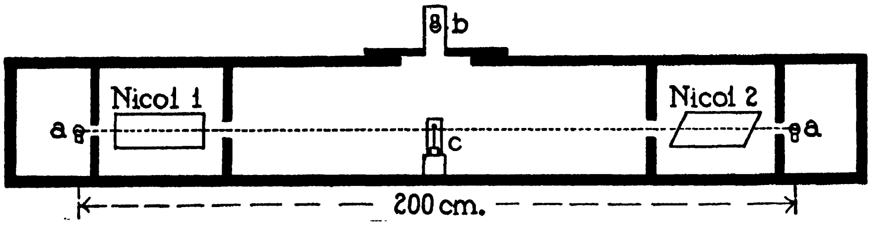
An explanation has been given of the greater absorption of light in the farther half of a single, cylindrical cell illuminated in air with parallel light from one side (Castle, 1933 *b*). Measurements of light paths within two halves of a cylindrical lens in air show that a greater total distance is traversed by the refracted rays in the far half than in the near half. The explanation is based largely on the idea that the primary action of light is on the cell protoplasm rather than on the wall. This interpretation has been tested in the experiments described in this paper, by making use of differences in the phototropic effectiveness of different planes of polarized light.

II

The upright sporangiophore of *Phycomyces* placed between two sources of light opposed at 180° is remarkably sensitive to differences in intensity between the two beams. Massart (1888) found that the cells responded to an intensity difference between the two sides of 18 per cent; Castle (1931) found a differential sensitivity of about twice Massart's value, in the vicinity of 8 per cent. Response is manifested by an inclination to and growth toward the more intense light. The cell does not reach a true position of equilibrium with reference to the two beams, and it is therefore best used as a "null" indicator, to show equal effects on opposite sides.

In the present experiments, two opposed beams of light plane polarized at right angles to each other were used. With reference to the vertically growing sporangiophores, one beam of light was polarized horizontally, the other vertically.

As shown in Text-fig. 1, two 3 candle power 6 volt automobile lamps were set up 2 meters apart and run in series on a 12 volt direct current line from the central storage battery of the Biological Laboratories. Voltage fluctuations thus tended to affect both lamps equally. A large Nicol prism was interposed in each beam, and adjusted so that the transmitted beams were plane polarized at right angles to each other, one vertically, the other horizontally.



Text-Fig. 1. Side view of the apparatus used to obtain opposed beams of polarized light. *a,a*, 3 candle power 6 volt automobile lamps run in series on a 12 volt line. *b*, movable lamp used to obtain vertical growth of cells before an experiment. *c*, culture of *Phycomyces* in a glass cell. The axes of the two Nicols are at right angles, so that the left beam is polarized horizontally, the right vertically.

A culture of *Phycomyces* was placed in a rectangular glass cell on a movable block at a particular place on the optical bench, and by means of a small electric lamp directly above the culture, sporangiophores were caused to grow up through a slit in the metal cover of the culture vessel. When the cells were at the proper stage of development, the overhead light was put out, and the lamps illuminating the cells from opposite sides with polarized light were turned on. Undisturbed growth was allowed to continue for 3 hours, then a photograph was taken of the cells from the side. Deviations from the vertical became more marked if the experiment continued for longer times. By using different cultures and varying the position on the optical bench, a region of phototropic balance was found where cells were either "indifferent," bending toward neither one side nor the other, or where approximately equal numbers bent in each direction. The conditions for equal phototropic effect of light polarized in each plane were determined by measuring the relative intensity of each beam at this region, using a Weston photronic cell and a Leeds and Northrup type "R" galvanometer with a 50 ohm shunt. Since the intensities of light were low, the photocell method proved the only satisfactory means of measurement. To avoid errors due to changes in the sensitivity of the photocell, the *ratio* of galvanometer deflections obtained from each beam at a given point on the optical bench was obtained. The measurements are expressed in terms of this intensity ratio, which proved reproducible to within

2 per cent. The sensitivity of the photocell to different planes of polarization was tested by rotating it in a beam of polarized light. No significant difference in the galvanometer deflection was observed. Any polarization effect in the photocell must amount to less than 1 per cent.

The end-point for equal and opposite phototropic effect is not as sharp as might be desired, for several reasons: (1) a positively phototropic organism is not in a condition of stable equilibrium under the circumstances of the experiment, since a random movement may place it under the sole orienting influence of either light source to the exclusion of the other. Furthermore, with equal phototropic stimulation on opposite sides, there is nothing but a weak negative geotropism to keep the cells upright in a plane perpendicular to the axis of the optical bench. Angular deviations of the sporangiophores from the vertical in this plane will alter, cancel, or reverse any differences between the effects of the two oppositely polarized beams. An experiment must therefore not be continued for such a long time that deviations from the vertical become prominent. (2) Until a certain intensity difference is reached between the two sides, no differential growth is perceptible. Massart found the critical difference to be 18 per cent, but this estimate is certainly too high. For the present experiment, this means that a definite zone will be found within which approximate phototropic balance prevails. (3) Phototropic balance is achieved by equating two different *kinds* of light, having different distributions of intensity within the cell. It is assumed that each half of the cell in effect summates the light absorbed within it, irrespective of the particular place of absorption. If this assumption is not completely justified, there will be room for more specific phototropic effects in the action of light polarized in different planes. Such effects, if existent, would complicate the conditions for phototropic balance.

III

The critical experiment consists in placing a culture at a position where the intensities of the two opposed beams are equal, differing only in plane of polarization. Plate 1, Fig. 1 *b*, shows a typical photograph taken at the end of such a test. The mature, actively growing cells have almost all bent to the left, showing that light which is polarized horizontally is phototropically more effective than light of equal energy polarized vertically. Examination of the other photographs of Plate 1, Fig. 1, confirms this finding, and shows that for equal phototropic effect the beam polarized vertically has to be 10 to 15 per cent more intense than the beam polarized horizontally. It is clear from these typical records that a more precise statement of the results is not justified, yet that a real difference exists in the effectiveness of the two beams.

Light polarized horizontally will undergo a smaller reflection loss at most angles of incidence on the cell surface than light polarized vertically. The loss in each case may be computed from Fresnel's formulae. For light polarized horizontally,

$$I_{\text{reflected}} = \frac{\tan^2 (i - r)}{\tan^2 (i + r)} I.$$

For light polarized vertically,

$$I_{\text{reflected}} = \frac{\sin^2 (i - r)}{\sin^2 (i + r)} I,$$

where

I = incident intensity
i = angle of incidence
r = " " refraction

For both planes of polarization, when $i = 0$

$$I_{\text{reflected}} = \left(\frac{n - 1}{n + 1}\right)^2 I$$

where

n = refractive index of the cell surface

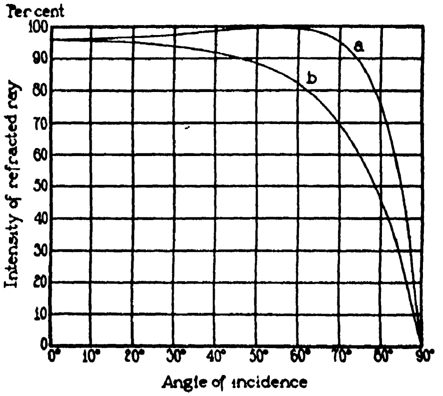
The percentage reflection losses of representative rays incident on the cylindrical surface of the cell at angles ranging from 0° to 90° were computed for both planes of polarization, and are given in Table I. In Text-fig. 2 the corresponding intensities transmitted into the cell are plotted against the angles of incidence. It is evident that in the case of light polarized horizontally, a large proportion of the rays incident on the cell at angles around 50° are refracted into the cell with little loss of intensity. As previously shown (Castle, 1933 *b*), it is especially these more tangential rays which have a long path in the back half of the cell relative to the front half. Consequently, greater relative absorption of light will take place there than in the case of light of equal intensity polarized vertically.

The magnitude of the difference which might be expected may be estimated by formulating the conditions necessary for phototropic balance. The basic assumptions and the general procedure of the method have already been described (Castle, 1933 *b*), and will not be detailed here. Use of polarized light is assumed only to alter the

TABLE I

Reflection loss and relative absorption in each half of a cylindrical cell of light plane polarized as described. l_1 and l_2 are taken from Castle (1933 *b*).

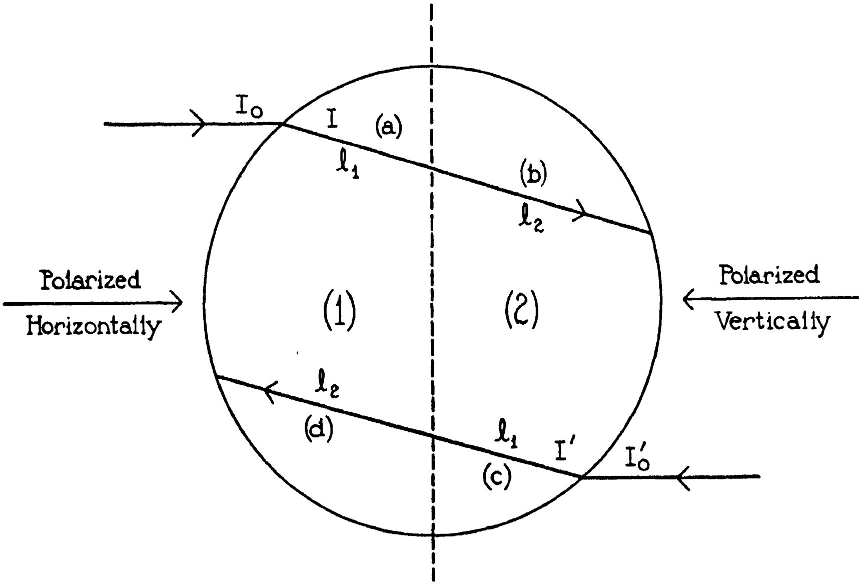
Angle of incidence	Sin i	Intensity loss by reflection		Intensity of refracted beam		Relative length of light pathway		$I \times l_1$	$I \times l_2$	$I' \times l_1$	$I' \times l_2$
i		Horizontally polarized	Vertically polarized	Horizontally polarized I	Vertically polarized I'	In front half of cell l_1	In back half of cell l_2	*a*	*b*	*c*	*d*
degrees		*per cent*	*per cent*	*per cent*	*per cent*						
0.0	0	4.0	4.0	96.0	96.0	1.000	1.000	96.0	96.0	96.0	96.0
11.7	0.203	3.7	4.2	96.3	95.8	0.980	1.000	94.4	96.3	93.9	95.8
23.8	0.404	3.1	5.0	96.9	95.0	0.925	0.992	89.6	96.1	88.0	94.2
37.0	0.602	1.8	7.0	98.2	93.0	0.816	0.985	80.1	96.7	76.0	91.6
44.5	0.701	0 9	9.0	99.1	91.0	0.739	0.990	73.2	98.1	67.2	90.1
53.4	0.803	0.1	13.1	98.9	86.9	0.629	1.010	62.8	100.8	54.7	87.8
64.3	0.901	1.1	21.3	98.9	76.9	0.478	1.045	47.3	103.3	36.7	80.4
72.3	0.953	6.6	34.0	93 4	66.0	0.352	1.105	32.9	103.2	23.2	72.9
90.0	1.0	100.0	100.0	0	0	0	1.380	0	0	0	0



TEXT-FIG. 2. Intensity of refracted rays for different angles of incidence, computed from Fresnel's formulae. *a*, incident beam polarized horizontally; *b*, incident beam polarized vertically. The index of refraction of the cell surface is taken as 1.5, that of air as unity.

amount of surface reflection and thus the relative intensities of particular rays within the cell. The further assumption is implicit that the effect of continuous light of low intensity in producing phototropism is directly proportional to the intensity.

Though it has been shown that light is refracted through these cells as if they had a mean refractive index of 1.38, in treating surface reflection the refractive index of the reflecting surface should be used. For the chitinous cell wall a value of $n = 1.50$ was used in the present calculations, although the light paths inside the cell were considered as within a cylindrical lens of refractive index 1.38. Reflection losses at the internal interface wall/protoplasm were neglected for the sake of simplicity.



Text-Fig. 3. Diagrammatic cross-section of a cell illuminated from opposite sides with polarized light. Only one ray in each beam is shown. Detailed description in the text.

Let Text-fig. 3 represent a cross-section of a cell illuminated with parallel light from two sides, the beam on the left being polarized horizontally, that on the right polarized vertically. For simplicity only one ray in each beam is represented. Let the cell be considered in terms of the two halves, (1) and (2). Furthermore, let

I_0 = intensity of incident ray polarized horizontally
I'_0 = " " " " " vertically
I = transmitted intensity of ray polarized horizontally

I' = transmitted intensity of ray polarized vertically
a = absorption of horizontally polarized ray in (1)
b = “ “ “ “ “ “ (2)
c = “ “ vertically “ “ “ (2)
d = “ “ “ “ “ “ (1)

Also, for any ray let

l_1 = length of light pathway in front half of cell[1]
l_2 = “ “ “ “ “ back “ “ “

The condition for phototropic balance is simply that the total absorption in the two halves must be the same, or that for all rays

$$\Sigma(a + d) = \Sigma(b + c)$$

The evaluation of a, b, c, and d is greatly simplified if the absorption coefficient, α, is regarded as infinitely small. If this assumption is made, the usual exponential form of the absorption law can be dispensed with, and relative absorption written equal to the product of intensity and length of absorbing path. Thus on this basis

$$a = I \times l_1 \qquad c = I' \times l_1$$

$$b = I \times l_2 \qquad d = I' \times l_2$$

The validity of this simplification depends on the exponent in the absorption law being small, implying either a thin absorbing layer or a small absorption coefficient, or both. Probably both of these conditions hold in the cell of *Phycomyces*. In any case, the following solution is for the limiting case of zero absorption. See Table I.

To obtain the summated values of a, b, c, and d for all rays, graphic integration is carried out as previously described (Castle, 1933 *b*).

$$\Sigma(a) = 2\int_0^1 Il_1 d \sin i \qquad \Sigma(d) = 2\int_0^1 I'l_2 d \sin i$$

$$\Sigma(b) = 2\int_0^1 Il_2 d \sin i \qquad \Sigma(c) = 2\int_0^1 I'l_1 d \sin i$$

The areas under the curves in Text-figs. 4 and 5 represent the relative amounts of absorption from two polarized beams of unit intensity incident as described. Table I gives the data from which the curves were made. It will be noted that the ratio of absorption in the two halves of the cell is different in the cases of the two oppositely polarized beams.

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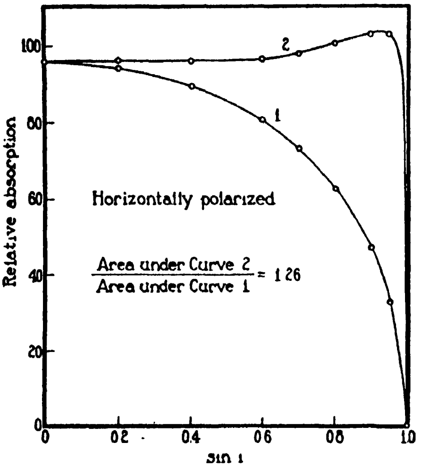
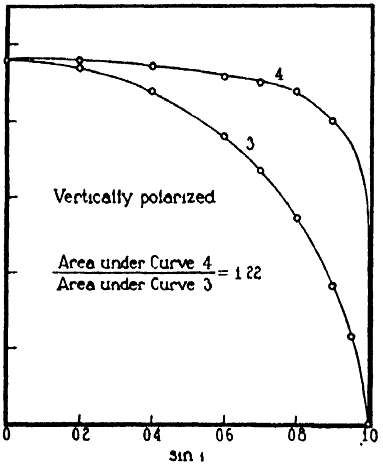
[1] “Front” half of the cell is here used to denote that half of the cell through which any incident ray first passes.



TEXT-FIG. 4



TEXT-FIG. 5

TEXT-FIG. 4. Plots showing relative absorption in front and back quadrants of the cell for different angles of incidence. The incident light is parallel and *polarized horizontally*. Twice the area under Curve 1 represents the total absorption in the front half of the cell; twice the area under Curve 2 the absorption in the back half.

TEXT-FIG. 5. Plots showing relative absorption in front and back quadrants of the cell for different angles of incidence. The incident light is parallel and *polarized vertically*. Twice the area under Curve 3 represents the total absorption in the front half of the cell; twice the area under Curve 4 the absorption in the back half.

Considering I_o as constant and equal to 1, what is wanted is the value of I'_o which will fulfill the condition

$$\Sigma(a + d) = \Sigma(b + c)$$

I_o' is therefore allowed to increase, which means that the areas under the curves in Text-fig. 5 will simply be multiplied by whatever value of I_o' is used. Table II shows that I_o' must increase to between 1.20 and 1.30 before the desired conditions are fulfilled.

This means that for phototropic balance the intensity of the vertically polarized light must be more than 20 per cent greater than that of the horizontally polarized light. The experimentally found figure was between 10 and 15 per cent. Considering the end-point of the experiment, it is clear that the difference between these values is not significant. The agreement could be made better if a small, finite value of absorption coefficient were used in the computations.

The magnitude of the difference found between the effects of vertically and horizontally polarized light can therefore be completely accounted for in terms of the suggested mechanism of absorption. This does not prove that the assumptions underlying the explanation are correct. Verification of the expected polarized light effect is, however, circumstantial evidence in favor of the suggested explanation.

TABLE II

Ratio of light absorbed in two halves of a cell illuminated from opposite sides with polarized light as described in the text. The incident intensity of the horizontally polarized beam is unity; that of the vertically polarized beam (I_o') is allowed to increase until the conditions for phototropic balance are met.

I_o', incident intensity of vertically polarized light	$\frac{\Sigma(b+c)}{\Sigma(a+d)}$
1.00	1.021
1.10	1.010
1.15	1.006
1.20	1.001
1.30	0.993

IV

A few tests have been made of the possible specific action of plane polarized light on living organisms, largely with negative results. Most conspicuously, the use of polarizing optical instruments implies that the human eye registers the intensity of light irrespective of its plane of polarization. Crozier and Mangelsdorf (1923) tested the phototropic efficiency of plane polarized light on several arthropods, and found no difference between it and non-polarized light of equal intensity. Macht (1927) reported that seedlings of several different kinds of plants grew faster in polarized light than in non-polarized light of the same intensity. His published data do not warrant this conclusion. He measured and summated the growth of the roots of different seedlings, usually twenty in number, half of which grew in ordinary and half in plane polarized light. In every case the total growth was numerically greater in the polarized light. Due to the extreme variability in the growth rate, the whole question is whether the numerical differences found are statistically significant. Computation of the probable errors of the differences in the case of Macht's squash seedlings shows that the differences are less than twice the probable errors of the differences. Similar computations for his *Lupinus* seedlings show differences ranging from less than one to four times the probable errors of the differences. Moreover, since the experimental conditions for securing light of identical spectral composition in the two experimental chambers were not rigorous, the possibility of a small consistent difference in spectral quality of illumination is not excluded.

The present experiments demonstrate a difference in the effect of light depending on its plane of polarization with reference to the axis of the cell. The difference which is found can be wholly accounted for by differences in the reflection losses at the cell surface and consequently in the relative intensities of certain rays of light within the cell. There is no need to postulate a more specific effect of plane polarized light on the growth processes of the cell. The difference which might be expected between the effects of polarized and unpolarized light has not been determined. It should be smaller than the effect measured above, and its detection more difficult.

I am indebted to Mr. William Arnold for suggesting the use of polarized light in these experiments and for many helpful discussions.

SUMMARY

For the growing cell of *Phycomyces*, a difference in the phototropic effect of light is described depending on its plane of polarization with reference to the axis of the cell. The difference which is found is primarily due to differences in the reflection losses at the cell surface. The magnitude of the effect approximates that deduced from the theory of phototropism suggested for this system. No specific effect

of plane polarized light on the growth processes of the cell need be postulated.

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EXPLANATION OF PLATE 1

FIG. 1. *a*, *b*, *c*, *d*, *e*, and *f* are photographs, taken at the end of 3 hours, of separate experiments in which cultures of originally straight cells were placed singly between two sources of light, the left polarized horizontally, the right vertically. The relative intensities of the lights on the two sides are expressed by the ratio given at the bottom of each plate. The top half of each photograph corresponds to the region where the intensities were measured, and where the mature, growing sporangiophores were. The degree of crowding in a culture is exaggerated, since the cells grew through a slit 2 cm. long perpendicular to the plane of the paper, and are here seen superimposed in silhouette. Real crowding and shading may be seen in the lower half of *a*. Note (1) that in *b* where the intensity ratio is nearly 1:1 the cells bend definitely to the left, and (2) that *d* and *e* represent approximate phototropic balance.

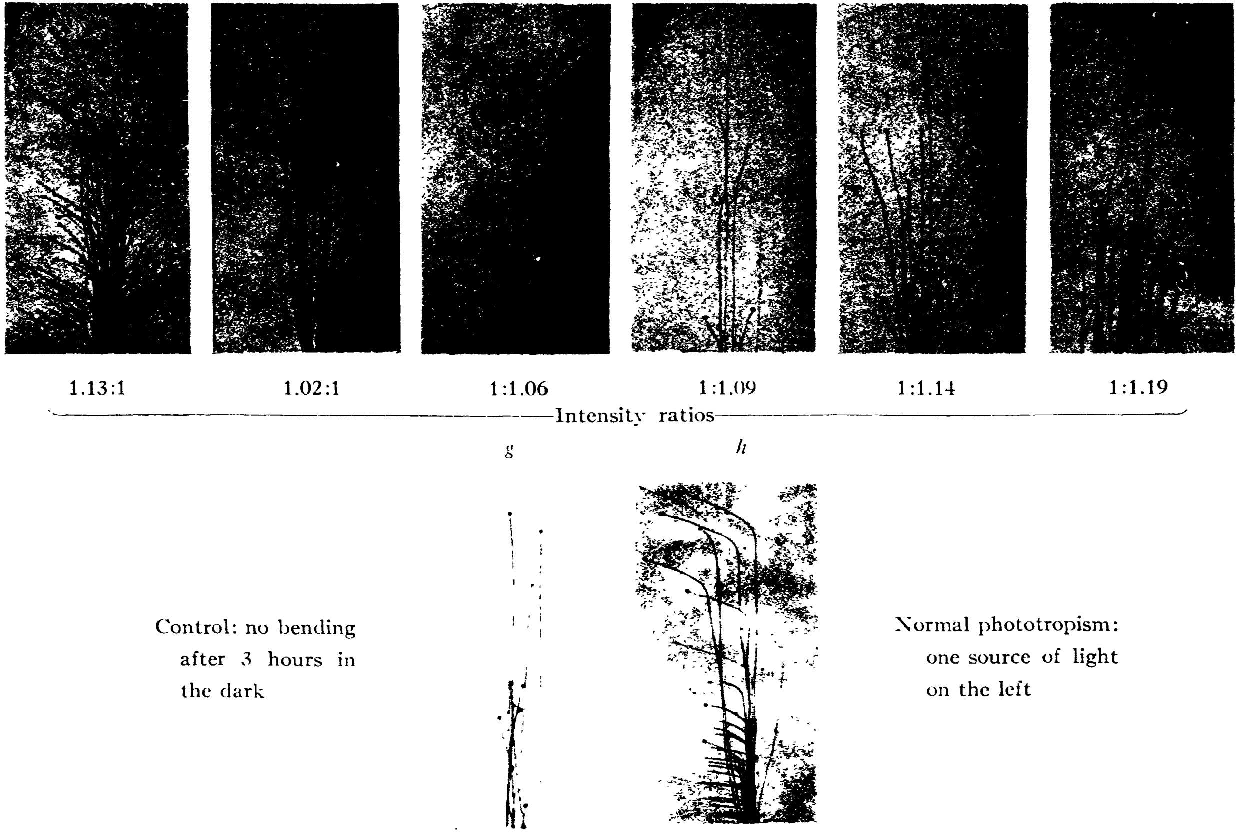
1.13:1
1.02:1
1:1.06
1:1.09
1:1.14
1:1.19
Intensity ratios
g
h
Control: no bending after 3 hours in the dark
Normal phototropism: one source of light on the left

FIG. 1

(Castle: Phototropic effect of polarized light)

THE GROWTH AND DURATION OF LIFE OF CELOSIA CRISTATA SEEDLINGS AT DIFFERENT TEMPERATURES

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Papers on the temperature relations of seedlings grown in darkness at several constant temperatures may be classified according to the length of the growing period during which measurements were made. Sierp (18), Silberschmidt (19), and Hamada (7) measured the lengths of oat coleoptiles throughout the grand period of growth, obtaining much the same results, although it is to be noted that arithmetical errors in Sierp's table (18, p. 442) apparently led him to erroneous conclusions as to the position of the optimal temperature. Silberschmidt (19) carried out experiments on pea and rice seedlings also; his data and his discussion are the most important recent contribution to this subject. Rudolfs (16) made similar measurements on bean seedlings but unfortunately he did not publish his numerical results. Pearl, Edwards, and Miner (14) reported the results of such tests on *Cucumis melo* seedlings. The data to be presented in this paper fall in the same category. In all these experiments the plotted growth data for each constant temperature fall along S-shaped curves of the logistic type. Both the final heights of the seedlings and the duration of growth, and consequently the time rate of growth, are influenced by temperature. Usually the temperature that induces the most rapid growth at first is somewhat higher than the one which finally yields the tallest seedlings.

Two important experiments extended over only a part of the growth cycle. Lehenbauer's (10) measurements of the growth of maize seedlings were made at nearly thirty constant temperatures and his data were the first to show a change in the optimal temperature as growth progressed. Unlike Lehenbauer's data, which showed an increase in the optimal temperature with time, Talma's (20) experiments on *Lepidium sativum* roots showed a decrease in the optimal temperature with time.

Measurements confined to the first stages of seedling growth were reported by Sachs (17) in 1860 and as a result of his work the papers of de Vries (22), Koeppen (9), and Haberlandt (6) appeared within a few years. All used seedlings of crop plants. Much later Newcombe (12) carried out experiments on the seedlings of fifteen species, mostly cultivated grasses and cereal crop plants. So far as they go, his data for *Lepidium sativum* confirm Talma's work. Leitch (11) and Cerighelli (2) presented data on the temperature relations of pea seedlings; Gericke (4) made tests on the early growth of wheat seedlings, and Fawcett's (3) tests on citrus seedlings belong in this class. Vogt (21) made measurements on the final length of oat coleoptiles grown in darkness at a number of constant temperatures and found an unusually low optimal temperature (12.8°C).

In the later work in this field less attention is paid to the temperature optimum[1] than formerly, principally because it is alleged to change as growth progresses. Sierp (18) and Silberschmidt (19) centered their attention principally on the grand period of growth and more than their predecessors regarded the series of constant temperatures they tested as a convenient means for creating a graded series of environments in which seedling growth could be studied, and this is the treatment to be followed here.

Material and Methods

Celosia cristata is a member of the Amaranthaceae extensively grown in flower gardens under the name of cockscomb. When grown on agar in darkness, as in these experiments, the seedlings develop a primary root which penetrates the agar to a depth of 2–3 cm. and which forms no lateral roots. The hypocotyl is curved at the upper end so that the cotyledon hangs downward for the first few days of growth and then it straightens, so that by the time growth has ceased the long, narrow, and reddish yellow cotyledons point upwards. The epicotyl does not develop under these conditions. The length of the hypocotyl seems to be a satisfactory measure of growth; the hypocotyl is cylindrical, and since there is very little increase in the diameter of the organ this measurement also may be regarded as proportional to the volume of tissue produced, and may be taken as an index of growth *yield*. The only organ competing with the hypocotyl for the food stored in the cotyledons is the primary root; this runs through a grand cycle of growth just as does the hypocotyl and terminates its growth a little earlier.

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[1] For a discussion of the sense in which optimum temperature for growth is used in our work in this laboratory, including the present paper, see Pearl, Edwards, and Miner (14).

The technique used in these experiments was, in general, that which has become standard in many years of seedling work in this laboratory (see Pearl (13), and earlier papers there cited, and also Gould, Pearl, Edwards, and Miner (5)).

Seeds of about the same size were sorted into lots of 22, having a combined weight of about 0.0195 gm., each lot being used for the experiments at one constant temperature. After being soaked in 1:1000 $HgCl_2$ solution the seeds were rinsed once and soaked for 3 hours in sterile distilled water. For germination and growth they were transferred to individual culture tubes 15 cm. long and about 1.6 cm. in internal diameter and distributed among constant temperature cases maintaining six temperatures between 14.5° and 40.5°C. The tubes were kept in darkness and the hypocotyl length was measured daily in red light.

After the rate of elongation had fallen below an average rate of about 0.1 mm. per day measurements were discontinued but the seedlings were kept at the same temperatures and observed regularly to ascertain the beginning of death of the hypocotyl of each seedling. The first visible signs of death of the hypocotyl were the appearance of a translucent zone or the shrinkage of the upper portion. In some specimens the cotyledons shrunk before the hypocotyls showed any morbid symptoms. At 40° blackening of the seedlings occurred at death and the symptoms in general differed from those of the other cultures so that the recorded data are not quite comparable in this one respect.

RESULTS

Growth

Table I shows the mean lengths of *Celosia* hypocotyls for various intervals after planting at the six constant temperatures tested. The cultures exposed at 14.5°, 20°, 30°, and 40.5° were first examined 12 hours after planting and at 24 hour intervals thereafter. The 25° and 35° cultures were first examined 18 hours after the seeds were placed in the culture tubes and subsequently at 24 hour intervals. Observations were also made 24 hours after planting at 30° and 35°. Besides the main series of tests which was begun in January, 1933, two temperatures, 25° and 35°, were tested in the spring of 1930; these data are presented in Table I for comparison with the other series but they were not used in the preparation of the graphs. These two sets of data for these temperatures appear to show satisfactory

TABLE I

Mean Heights of Celosia cristata Seedlings at Different Intervals after Planting

Interval after planting	Temperature				Interval after planting	1933 data	1930 data	1933 data	1930 data
	14.5°	20°	30°	40.5°		25°		35°	
days	*mm.*	*mm.*	*mm.*	*mm.*	*days*	*mm.*	*mm.*	*mm.*	*mm.*
1.0	—	—	0.9	—	1.00	—	—	2.0	—
1.5	—	—	3.0	1.5	1.75	1.1	1.1	5.2	5.4
2.5	—	—	11.4	6.2	2.75	6.1	5.7	16.4	11.6
3.5	—	—	21.9	10.1	3.75	13.4	13.7	26.5	25.2
4.5	—	1.3	29.7	11.7	4.75	20.2	21.1	31.5	30.3
5.5	—	4.0	34.0	12.3	5.75	26.0	24.8	33.5	31.9
6.5	—	8.4	35.7	12.7	6.75	29.1	26.5	34.1	32.5
7.5	—	12.8	36.2	—	7.75	30.7	27.1	—	32.7
8.5	0.7	17.0	36.3	—	8.75	31.6	27.7	—	—
9.5	1.7	20.8	—	—	9.75	31.7	—	—	—
10.5	2.6	23.0	—	—	—	—	—	—	—
11.5	3.7	24.6	—	—	—	—	—	—	—
12.5	5.6	26.2	—	—	—	—	—	—	—
13.5	7.3	26.8	—	—	—	—	—	—	—
14.5	9.4	27.4	—	—	—	—	—	—	—
15.5	11.3	27.7	—	—	—	—	—	—	—
16.5	13.9	—	—	—	—	—	—	—	—
17.5	15.3	—	—	—	—	—	—	—	—
18.5	17.0	—	—	—	—	—	—	—	—
19.5	17.7	28.3	—	—	—	—	—	—	—
20.5	18.7	—	—	—	—	—	—	—	—
21.5	19.3	—	—	—	—	—	—	—	—
22.5	19.9	—	—	—	—	—	—	—	—
23.5	20.0	—	—	—	—	—	—	—	—

agreement considering that commercial seed produced in different seasons were used in the two series of tests.

Fig. 1 shows the growth curves that result when these data are plotted, and brings out the considerable differences in height and growth rate associated with different temperatures. For a considerable part of the growth period the seedlings cultured at 35° were taller than at any other temperature and it was not until comparatively late in the growth cycle that they were surpassed by the 30° seedlings. At 25° growth was slower and the final height was a little less than at 35°. At 20° the beginning of measurable elongation was delayed and both growth rate and final height were less than at 25°. There is a much longer latent period before growth commences at 14.5° and growth rate and final height are still less. The irregularities of the 14.5° curve are probably due to difficulties of measurement; the curvature of the agar surface tended to conceal the base of the hypocotyl.

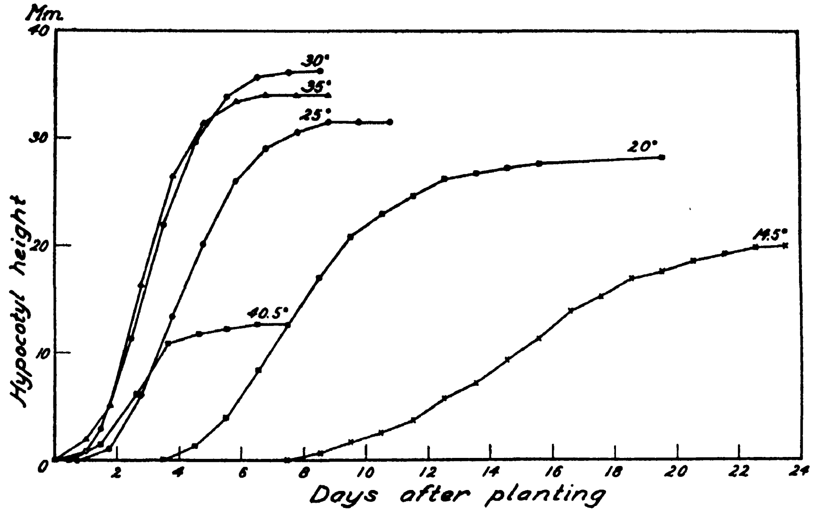

FIG. 1. Growth curve of *Celosia cristata* hypocotyls grown in darkness at six constant temperatures.

In these respects the behavior of *Celosia* is very similar to that of *Cucumis melo* seedlings grown at these temperatures with the same treatment (14).

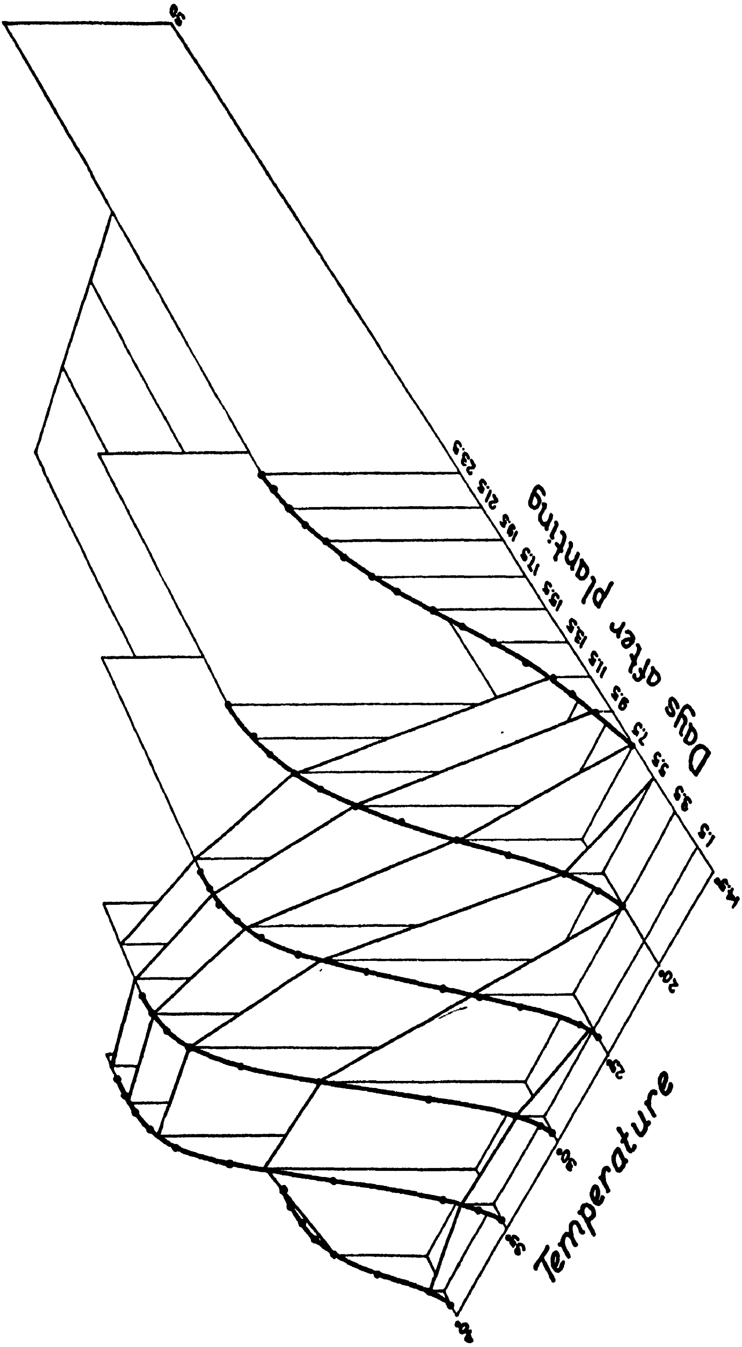

FIG. 2. Three dimensional diagram showing the growth and duration of life of *Celosia cristata* hypocotyls at six constan emperatures. Dots indicate observed heights of hypocotyls.

At 40.5° growth abnormalities were observed; many hypocotyls were bent or twisted and some underwent very little elongation, and the roots, for the most part, either lay on the surface of the agar or penetrated it so shallowly that they provided very little anchorage. Six of the seeds were planted near enough the wall of the tube so that the hypocotyl could obtain mechanical support by resting against the wall and these six were used for growth measurements. It should be emphasized that the appearance of the measured hypocotyls, and of the ones which were so coiled that measurement was impossible, was not greatly different from that of seedlings grown at lower temperatures, apart from their shortness. It appears that the hypocotyls were able to elongate, although at a very low rate compared with the 35° cultures, and to grow upward from whatever position in which they found themselves, but the failure of the roots to function properly left the hypocotyls without any anchorage. Any normal geotropic response of the hypocotyl would alter the center of gravity of the seedling making it fall into another position from which another geotropic response could be made only by altering the direction of growth.

The three dimensional diagram shown in Fig. 2 has been constructed from the data of Table I according to the same plan, and by the same methods, as the corresponding diagram in our *Cucumis* paper (14) except that in the present case the part of the curve which approaches the upper asymptote has been extended to show the total duration of life (Table II).

An inspection of the transverse planes of the diagram, which connect culture periods of the same duration, reveals a gradual apparent shift of the temperature at which growth is temporarily going on at the most rapid rate, from 35° at the beginning of growth to 30° by the time growth ceased. In other words, after a high initial growth rate the 35° seedlings elongated more slowly than the 30° ones. This will appear more clearly as the data are analyzed quantitatively. The same sort of shift of the growth rate with temperature as growth progresses has been found by Lehenbauer (10), Talma (20), Silberschmidt (19), and Pearl, Edwards, and Miner (14).

Having seen graphically the general course of events in the experiments we may now proceed to their analysis along the same lines as were followed in our study of *Cucumis melo* seedlings. If the total average growth rate at each temperature is computed by dividing the mean total yield (length of hypocotyl) by the time from planting to cessation of growth, it is found that in *Celosia*, just as in *Cucumis*, the relation between temperature and time rate of growth is parabolic over the observed range of these experiments.

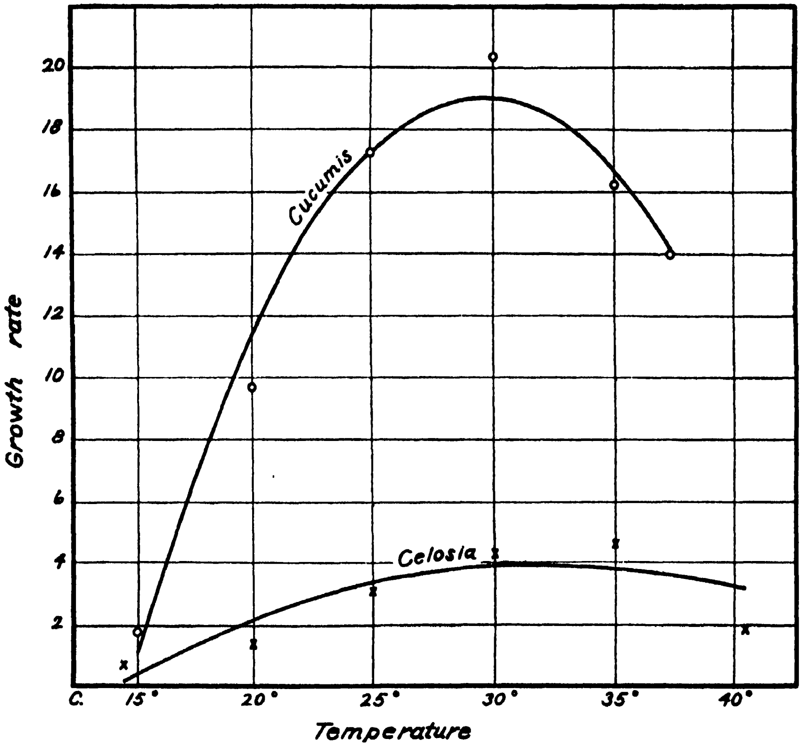

FIG. 3. The relation between temperature and mean total growth rate in the seedlings of (*a*) *Celosia cristata*, and (*b*) *Cucumis melo*. The observations are given by crosses in the case of *Celosia* and by circles in the case of *Cucumis*. The smooth curves are the graphs of the respective parabolas, fitted by least squares.

Fitting by least squares, the equation is

$$R = 0.898\,T - 0.015T^2 - 9.689, \qquad \text{(i)}$$

where R denotes mean total growth rate, and T temperature in Centigrade degrees.

In Fig. 3 this curve is plotted against the observations, and it is apparent that it describes the relations with reasonable accuracy, when the relatively small magnitude of the observational material is taken into account. For the sake of comparison the corresponding curve from our *Cucumis melo* study is plotted on the same diagram.

It is seen from Fig. 3 that while these two species follow the same general rule as regards the relation of growth to temperature under the specified experimental conditions, in that the mean total growth rate is lower as the temperature departs from the optimum in either direction, there is in one respect a striking difference between the two species. *Celosia cristata* seedlings are much less sensitive to a differential influence of temperature upon growth than are *Cucumis melo* seedlings. The *Celosia* curve is much flatter. In the case of *Cucumis* the mean total growth rate at the observed optimum temperature (30°) is roughly ten times as great as it is at 15°. On the other hand, in the case of *Celosia* the mean total growth rate at the observed optimum temperature (30°) is only about five times as great as at 15°, and even less at the probably true optimum temperature (from the fitted curve). In other words, *Celosia* seedlings are much less thermolabile than *Cucumis* seedlings.

While from the gross observations it would appear that the optimum temperature for growth rate was higher for *Celosia* than for *Cucumis,* actually if the differential coefficient for equation (i) is put equal to zero the result is

$$\frac{dR}{dT} = 0.89752 - 0.029444\,T = 0, \tag{ii}$$

and

$$T_{\text{opt.}} = 30.48°$$

This value is thus close to that reported in our *Cucumis* study (14), which was 29.74°.

The above analysis of the quantitative relations between temperature and growth follows the same lines as that of our *Cucumis melo* study (14). In the present case, as in that, we tried different postulates as to a single numerical measure of mean growth rate in a given temperature, and as to the separation of visible, measured growth of hypocotyl from the processes of germination. But in *Celosia,* just as in *Cucumis,* it was found that no difference in the essential results or conclusions resulted. For a detailed discussion of this matter the reader is referred to the *Cucumis* paper (14). There seems no point in repeating it here.

It has been noted above that in these experiments temperature appears to affect growth differently in different parts of the cycle. The observations on the point are given more precisely in Table II. The first set of percentages, given in the left half of Table II, state the approximate percentage of the final mean total yield (hypocotyl length) at each temperature which the plants in that temperature series have achieved at the end of the first quarter, the first half, and the first three-quarters of their own respective total growth periods. Thus at the end of the first half of their growth period the 14.5° plants had made 20.9 per cent of their total yield. The second set of figures, in the right half of Table II, shows the approximate percentage which the achieved yield in each temperature series was of the yield in the 30° series (30° being the observed optimum temperature for total yield) in one-quarter, one-half, and three-quarters of the total growth period. Thus it appears that the 20° plants at the end of half their growth period had produced only 76.9 per cent as much yield as the 30° plants in the first half of their cycle. The percentages in Table II are approximate because, in interpolating times and yields, we have assumed that growth proceeded at a constant rate between any two recorded observations in Table I. This is not strictly true, but the error is negligible for present purposes. In the computations germination, in respect of time, is counted as a part of growth.

TABLE II

Approximate Relative Hypocotyl Lengths at Stated Relative Times, and in Proportion to 30° Hypocotyl Lengths at the Same Relative Times

Percentages of total growth period	Percentages of own total growth						Percentages of own growth to 30° growth at same relative times					
	14.5°	20°	25°*	30°	35°*	40.5°	14.5°	20°	25°*	30°	35°*	40.5°
25	0	8.2	14.3	22.7	19.4	16.4	0	28.0	55.0	100	44.2	25.3
50	20.9	75.4	66.0	76.4	66.6	71.9	15.0	76.9	75.4	100	81.8	32.9
75	77.6	96.9	94.6	97.8	94 2	93.9	43.7	77.3	84.5	100	90.5	33.6
100	100	100	100	100	100	100	55.1	78.0	87.3	100	93.9	35.0

* 1933 data.

From Table II the following points may be noted.

1. The seedlings in these experiments did not in any case attain as much as 25 per cent of their total growth in the first quarter of their total growth period. Those in the 30° series came nearest to it.

2. When a half of the growing period had been completed the seedlings in all of the temperature series except at 14.5° had achieved 2/3 or more of their final total yields. At the end of the first three quarters of their total growth periods all of the seedlings had achieved more than 75 per cent of their total yields, and all of them except in the 14.5° series well over 90 per cent of the final total yields.

3. In *Celosia cristata*, under the conditions of these experiments, just as we have shown (14) to be the case in *Cucumis melo*, the greatest growth activity was concentrated in the second quarter of the growth cycle, except at the lowest (14.5°) temperature where the greatest growth per unit of time occurred in the third quarter of the cycle.

4. Except in the 14.5° series the amount of growth activity displayed in the fourth (final) quarter of the cycle was extremely small.

5. At all other temperatures tested, and at all parts of the growth cycle, the yield at any given comparable point in the cycle was less than that in the 30° series at the corresponding time. In other words, while, as has already been pointed out, maximum growth activity may occur at different parts of the cycle in different temperatures, the greatest growth of *Celosia cristata* seedlings as measured by yield occurs at 30°, not only in the cycle as a whole, but also in each of its equivalent parts, on a relative time scale. In the face of this fact it would seem to be an error, based upon insufficiently penetrating analysis, to speak of the optimal temperature shifting about during different parts of the cycle, at least in this case. The point in the matter, which is essential and appears to have been sometimes overlooked, is that biologically equivalent points in the growth cycle (and indeed in the life cycle generally) cannot be correctly apprehended or determined in terms solely of absolute (chronological) time units, but require some sort of relative time scale. Essentially the same point is involved in discussions of "physiological" *versus* "chronological" age.

6. *Celosia cristata* seedlings, under the conditions of the experiments, are generally speaking relatively more rapid growers in the first quarter of the cycle at all temperatures than *Cucumis melo* seedlings.

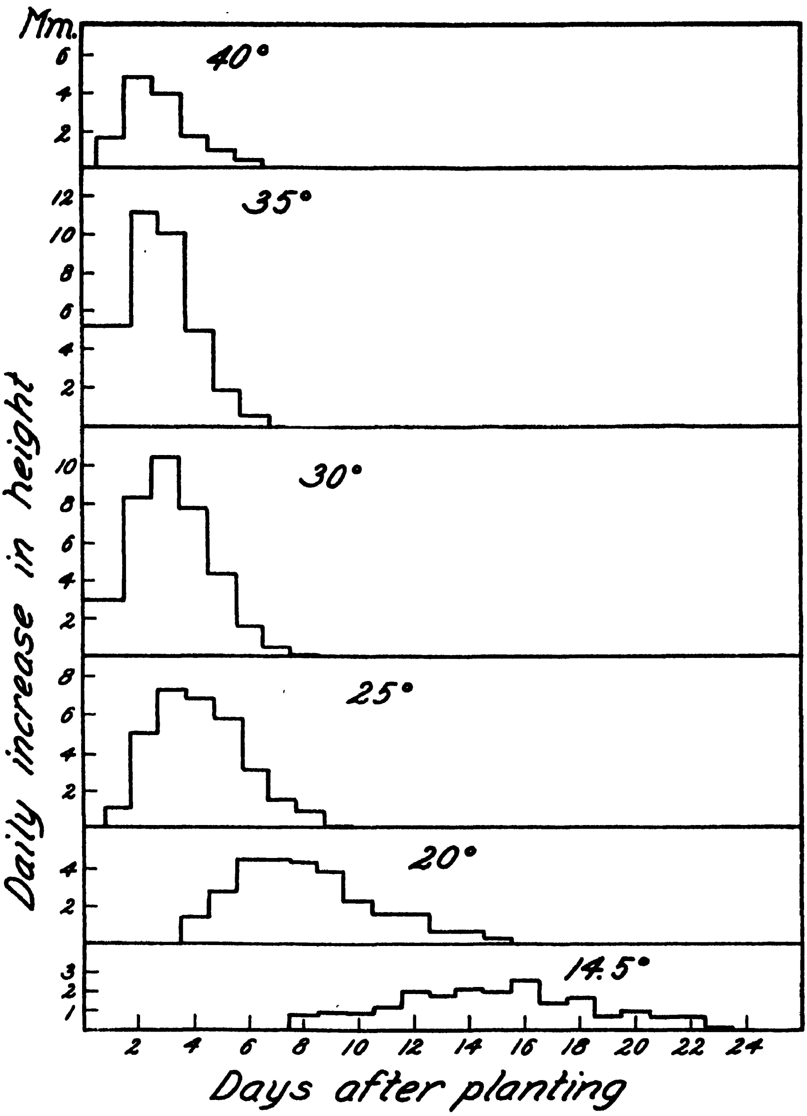

FIG. 4. Increases in height of *Celosia* hypocotyls during 24 hour periods. For 14.5°, however, 48 hour increments were used.

The points just discussed may be looked at in another way by examining the absolute growth increments in absolute (chronological) time in the several series. These are shown graphically in Fig. 4.

As one examines the graphs of the growth increments in turn, beginning with 14.5°, it is seen that there is a tendency for the period of most active elongation to come progressively earlier in chronological time units as higher temperatures are examined until 40.5° is reached where there is a delay, compared with 35°. This statement is true in two senses: (1) If one measures the time of occurrence of the peak of these graphs in terms of days after the time of planting, this relationship stands out clearly, and it is, in fact, the kind of relationship which would be expected on the basis of other work on the temperature relations of biological processes. (2) If the graphs are examined one by one to find when the period of most active elongation fell in relation to the whole growth period a similar kind of trend is encountered. At the lowest temperature the greatest growth rate came relatively late in the growth cycle, at 20° it came a little before the middle point of the period in which measurable growth occurred, and each higher temperature had the effect of inducing a larger and larger proportion of the total growth to occur during the first few days of growth. But in another way, there is a regular change in symmetry as one examines the graphs in turn. Both kinds of relationship have also been observed in *Cucumis melo* seedlings (14).

Fig. 3 shows nothing different from, or additional to, what is brought out in precise numerical terms in Table II. But inasmuch as the analysis of growth in terms of relative time given in Table II and the discussion following it is, so far as we are aware, novel, it has seemed to us advisable to insert Fig. 4 in order to exhibit the results in a form accordant with the conventional procedure in work upon growth in seedlings.

Duration of Life

In considering the durations of life of these seedlings, which are represented in Fig. 2 as extensions of the sigmoid growth curves, the experimental conditions need to be kept in mind. Since the seedlings were grown under aseptic conditions throughout, death was due to failure of the plant to be able to get further food material from the cotyledons, and not because of microbial attack. The only materials that were supplied to the plant during its lifetime were air, distilled water, and agar, and agar has been shown by earlier unpublished work not to be a source of food in sufficient amount to be either physically or chemically detectable or measurable. Since the seedlings were kept in darkness (except for brief exposure to red light during measurement) no chlorophyll developed and photosynthesis was impossible. Thus the seedlings were entirely dependent upon the food reserves laid down in the cotyledons and embryo, and since the same weight of seeds of a uniform size was used for each culture the six lots of seedlings began on an equal basis. Their durations of life, however, were quite different, as Fig. 2 and Table III show; the seedlings grown at 14.5°, for instance, lived five times as long as those grown at 35°, and disregarding for a moment the 40.5° cultures, the duration of life was inversely proportional to the temperature. Furthermore the data show that the intermediate period of the life cycle of these seedlings (period from end of growth to beginning of death) is lengthened in temperatures below the growth optimum and shortened in temperatures above the growth optimum. In other words the optimal temperature for duration of life in these experiments was the minimum temperature, within the range of observations. These observations are definitely and significantly confirmatory of the "rate of living" (13) theory of life duration. The rate of living, as manifested by rate of growth for example, is influenced by temperature, as inorganic chemical reactions are, and in consequence the time duration of life is altered in an orderly manner. When the time rate of growth is more rapid not only is the

TABLE III

Final Heights and Durations of Growth and of Life of Celosia cristata Seedlings at Various Temperatures

	Temperature							
	14.5°	20°	25°		30°	35°		40.5°
			1933 data	1930 data		1933 data	1930 data	
Final hypocotyl height, *mm*	20.0 ±0.5	28.3 ±0.9	31.7 ±0.6	27.7 ±1.3	36.3 ±0.6	34.7 ±0.9	32.5 ±0.7	12.7
Duration of growth, *days*	23.5	19.5	9.75	8.75	8.5	6.75	7.75	7.75
Duration of intermediate period, *days*	26.6	13.5	13.4	15.7	5.4	5	3.9	5.7
Duration of life (total), *days*	50.1	33.0	23.15	24.45	13.9	11.75	11.65	13.45
No. of seedlings	14	18	19	15	20	17	19	6

period of growth shortened, ***but so also is the duration of the intermediate period after growth has ceased, and the total duration of life.***

The inverse relation between growth rate and life duration described in the preceding paragraph is shown quantitatively in Table IV and Fig. 5. It is obvious that what we have called the "intermediate" period—that is the time from the end of growth to the beginning of death—is the crucial element in total life duration, from the theoretical point of view. Naturally if the growth period is lengthened, with consequently lowered growth rate, as in the low temperatures, that fact will of itself tend to lengthen total duration of life, which is the sum of growth period + intermediate period. Consequently we shall deal separately with duration of intermediate period and total longevity.

TABLE IV

Relative Magnitudes at Different Temperatures of Mean Total Growth Rate and the Duration of Various Portions of the Life Cycle in Celosia cristata Seedlings

Temperature	Relative observed growth rate	Relative duration of intermediate period	Relative duration of growth period	Relative total longevity
°C.	*per cent*	*per cent*	*per cent*	*per cent*
14.5	26.5	198.5	241.0	216.4
20	45.2	100.7	200.0	142.5
25	100	100	100	100
30	133.0	40.3	87.2	60.0
35	143.6	37.3	69.2	50.8
40.5	60.7	42.5	79.5	58.3

In Table IV the performance of the seedlings at 25° in respect of (*a*) mean total growth rate, (*b*) duration of intermediate period, (*c*) duration of growth period, and (*d*) total longevity, is taken as 100 per cent for each variable and the relative (percentage) values on this basis at each of the other temperatures tested are then set down.

It is evident that both total duration of life and the duration of the crucially important intermediate period, exhibit an inverse relation to the rate of growth. From 15° to 35° inclusive, where the mean total growth rate is increasing with rising temperature, total longevity and duration of the intermediate period decline. At 40.5° where the total growth rate falls, the durations rise. The rise at this high temperature is not, however, in either case anything like proportional to the relative decline in the growth rate, and too much stress should not be laid upon it, particularly in view of the abnormal character of the growth and the dying at this temperature. At 35° and temperatures below, the inverse proportionality between growth rate and duration of life is more precisely and regularly maintained. By slowing the growth rate to roughly a quarter of its value at 25° the total duration of life is about doubled.

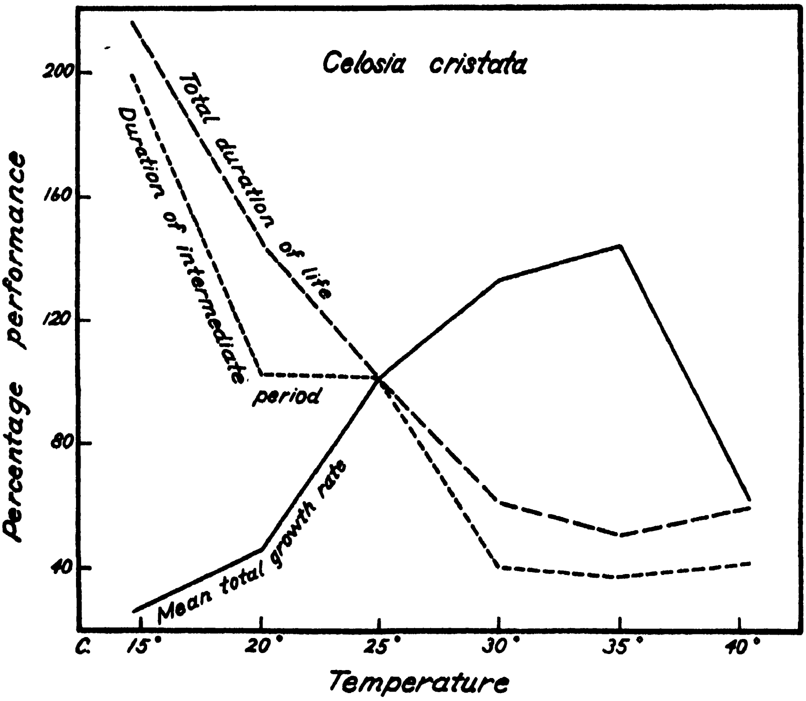

Fig. 5. Showing the inverse relation between mean total rate of growth and total duration of life, and duration of intermediate period of the life cycle.

In 1916 Rahn (15) sought to extend the reasoning of Blackman (1) and of Jost (8) and to account for limitations of size and duration of life on a chemical basis. He assumed: (1) The quantity of material capable of being transformed by a given quantity of enzyme is definitely limited, and this limits the quantity of material which an organism can metabolize during its lifetime. (2) Each enzyme is the product of a chain of reactions, whose links may have different temperature coefficients. Thus according to the temperature at which an organism is grown it may have a large or a small quantity of enzyme at its disposal, and may have a large or a small final size, or a long or a short duration of life. Rahn was careful to state that his speculation was "*ganz unbeweisbar*," and it does not seem to be particularly helpful in the consideration of the present data.

A complete and precise quantitative analysis of the inverse relationship between rate of growth and subsequent duration of life (and also total duration of life) in these seedlings would require more extensive data, and far finer temperature divisions than the present experiments furnish.

SUMMARY

Daily measurements of hypocotyl length were made on *Celosia cristata* seedlings cultured in darkness under aseptic conditions at six constant temperatures between 14.5° and 40.5°C. At 40.5° roots did not penetrate the agar and only the hypocotyls that were supported by the wall of the test tube could be measured.

The growth curves were of the generalized logistic type, but of different degrees of skewness. The degree of symmetry of the growth curves was influenced by temperature. At the lower temperatures the maximal growth rate came relatively late in the grand period of growth; at successively higher temperatures it came progressively earlier.

The mean total time rate of growth (millimeter *per diem*) was found to be a parabolic function of the temperature.

The maximum rate of growth was found from the curve to be at 30.48°C. The maximum observed rate of growth, and the maximum yield, were found to be at 30°C.

At all temperatures above 14.5° the maximum growth activity fell in the second quarter of the whole growth period. At all temperatures tested other than 30°, and at all parts of the growth cycle, the growth yield as measured by height of hypocotyl at any given equivalent point was less than at 30°.

The total duration of life of the seedlings, and the duration of life after the end of the growth period (intermediate period) were inversely proportional to the mean total growth rate. The observations on

Celosia cristata seedlings are thus in accord with the "rate of living" theory of life duration.

The optimal temperature for life duration is the minimum temperature, within the range of these observations.

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BOUND WATER IN MUSCLE

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Rubner (1922) has defined the bound water in a colloidal system as the water which does not freeze at −20°C. On this basis Rubner (1922) and Thoenes (1925) obtained large values ranging from 0.8 to 2 gm. of bound water in muscle per gm. of dry solid. The same definition has been applied by other investigators to various biological systems; it has, however, been criticised by Briggs (1931) and Heiss (1933) on the ground that in systems containing crystalloids, unfrozen free water (*i.e.* water acting as normal solvent) may be present at −20°C.

It seems preferable to define bound water as water which does not act as solvent. On this basis low values at 0°C. were calculated from the vapour pressure isotherm of frog's muscle (Brooks, 1933–34). At an activity of free water of 0.994 it was found that 0.3 gm. of water was bound by 1 gm. of dry solid. Hill (1930) had previously obtained by another method a still lower figure; this may be due partly to the higher temperature employed, namely about 16°C. These results, when compared with the degree of hydration of various proteins in solution, indicate that the bound water in muscle is mainly, if not wholly, accounted for by the water of hydration of the muscle proteins. It would be expected that the degree of hydration would be influenced by the activity of free water and the temperature.

An attempt has been made to calculate the amount of free unfrozen water in muscle at different temperatures below the initial freezing-point from the vapour isotherm at 0°C. The results indicate that a significant amount of free unfrozen water is present at −20°C. and that the system invariant point is considerably below this temperature. The total amount of unfrozen water at −20°C. gives therefore only a maximum figure for the bound water at that temperature. The large values obtained by Rubner and Thoenes can be attributed partly to this cause and partly, to an even greater extent in view of the later work of Heiss (1933) and Moran (private communication) on the freezing-point curve of muscle, to the considerable experimental difficulties of measuring unfrozen water at low temperatures.

The difference between the calculated amount of free unfrozen water in muscle at a given temperature and the amount of total unfrozen water experimentally determined by Heiss or Moran should give the bound water present. The values so obtained are of similar magnitude to those calculated from the vapour pressure isotherm at 0°C.

The Freezing-Point Curve of Muscle

In a "frozen"muscle in equilibrium at a temperature T' the activity of free water, a', is equal to the activity of ice at that temperature and is given by the equation (Lewis and Randall, 1923)

$$\log a' = -0.004211\,v - 0.0000022\,v^2 \tag{1}$$

where $v = 273 - T'$.

The amount of free water present in frog's muscle in rigor at 0°C. and at different activities of water has been calculated from the vapour pressure isotherm. The reference state of the muscle is taken as the water content at 0°C. (T'') and an activity of free water (a_0'') of 0.994. At this activity and temperature it was found that 100 gm. of muscle contained 19.9 gm. of dry solid, 74.3 gm. of free water, and 5.8 gm. of bound water. Let the weight of free water per 19.9 gm. of dry solid at the same temperature T'' and another activity of water a_1'' be m gm. Then if the activity of free water in a muscle is independent of the temperature the decrease of temperature v required to freeze out $(74.3 - m)$ gm. of free water from 100 gm. of muscle in the reference state is given by substituting a_1'' for a' in (1).

It cannot be assumed, however, that the activity of water in a muscle is independent of temperature. The relation between temperature and activity is given by the equation:

$$\frac{d \ln a}{dT} = -\frac{\bar{L}_1}{RT^2} \tag{2}$$

where $\bar{L}_1$ is the relative partial molal heat content of water in the muscle. For most solutions $\bar{L}_1$ is negligible unless the solution is concentrated or has an exceptionally large heat of dilution. It would be expected therefore for high values of a_1'' that the activity of water in a muscle would not alter greatly with temperature.

Lewis and Randall (1923) take $\bar{L}_1$ as a linear function of temperature[1] and integrating (2) at constant composition obtain the following equation for the change in activity of water with temperature:

$$\log a''_1 - \log a'_1 = x \tag{3}$$

where

$$x = -\bar{L}_{1(T'')}\cdot\frac{T'' - T'}{2.303\,RT''T'} + (\bar{C}_{p_1} - \bar{C}^{\circ}_{p_1})\left(T''\cdot\frac{T'' - T'}{2.303\,RT''T'} - \frac{1}{R}\log\frac{T''}{T'}\right) \tag{4}$$

and $\bar{C}p_1$ and $\bar{C}^{\circ}p_1$ are partial molal heat capacities of water.

The substitution of (3) in (1) gives the following equation,

$$\log a''_1 + 0.004211\,v + 0.0000022\,v^2 = x \tag{5}$$

where x is given by (4). If therefore at one temperature, T'', the free water content and the partial molal heat terms for water in muscle at different activities of water, a_1'', are known the required values of v (where $v = 273 - T'$) can be obtained from (5).

The partial molal heat terms for water in muscle are unknown, but when the crystalloid constituents of muscle are considered,[2] it is reasonable to assume that they do not differ greatly from the heat terms for water in a solution of a uni-univalent electrolyte, *e.g.* sodium or potassium chloride, of equivalent concentration. The concentrations of solutions of sodium chloride with activities of water at 0°C. corresponding to the activities used (down to $a_1'' = 0.798$) in the determination of the vapour pressure isotherm have already been calculated (Brooks, 1933–34). These values are given in Table I, together with the mean amounts of free and bound water in frog's muscle at 0°C. and at different activities of water. The calculated values of free water at $a'' = 0.85$ and 0.80 are less accurate than those at the higher activities but are probably of the correct order.

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[1] This assumption, according to Young (1933), is not strictly correct. It is sufficiently accurate for the present purpose, however.

[2] The proteins in muscle would not be expected to influence $\bar{L}_1$ unless the total water content of the muscle was lower than approximately 0.3 gm. of water per gm. of protein (*cf.* Rosenbohm, 1914; Moran, 1932).

The partial molal heat terms for water at 0°C. in the solutions of sodium chloride given in Table I were obtained from the data of Randall and Bisson (Lewis and Randall, 1923) at 25°C. These values, together with the corresponding values of a''_1, were substituted in (5), giving v (or $273 - T'$). The first column of Table II contains the molality of sodium chloride and the second column the corresponding value of v from (5).

It will be seen that this particular use of (5) gives simply the freezing-point of the solutions of sodium chloride in the first column. For comparison therefore the third column contains the experimental values of the freezing-points interpolated from those given in the International Critical Tables.[3] The fourth column gives values of v obtained on the assumption that $\bar{L}_1$ is zero; *i.e.*, by the direct substitution of a''_1 in (1). As would be expected this assumption does not lead to a large error in the calculated freezing-point except at low activities of water.

TABLE I

Dry Weight of Muscle = 19.9 Gm.

a''	Molality of sodium chloride	Equilibrium weight of muscle	Free water	Bound water
		gm.	*gm.*	*gm.*
0.9936	0.1969	100	74.3	5.8
0.9552	1.381	36.3	10.6	5.8
0.8904	3.138	30.5	4.7	5.9
0.8502	4.098	28.0	3.5	4.6
0.7984	5.215	26.8	2.8	4.1

The fourth column of Table I and the second (or third) column of Table II give the amounts of free unfrozen water remaining in 100 gm. of "frozen" frog's muscle (initially in the reference state) in equilibrium at v degrees below 0°C. These values are given below.

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[3] International Critical Tables, New York, McGraw-Hill Book Co., Inc., 1928, **4**, 258.

v	0.67*	4.7	11.6	16.0	21.9
Free water, *gm*	74.3	10.6	4.7	3.5	2.8

* −0.67°C. is the calculated value for the initial freezing-point of frog's muscle in rigor; the freezing-point of living frog's muscle is −0.42°C. Hill (1930) gives the osmotic equivalent of frog's muscle in rigor as 0.2 molal sodium chloride; this solution has a freezing-point of −0.68°C.

If the total unfrozen water at −21.9°C. is considered as bound and expressed as gm. of bound water per gm. of dry solid it will be seen that the value so obtained is approximately 2.8/19.9 = 0.14 gm. too high.

The proportion of free unfrozen water in beef muscle (in rigor) at a given temperature will be greater than in frog's muscle as the initial freezing-point is lower (−1°C. instead of −0.67°C.). Assuming that the water-binding capacity of the proteins in beef muscle at 0°C. is the same as in frog's muscle at 0°C. and making allowance for the differences in initial freezing-point and total water content (77 instead of 80 per cent) the amounts of free unfrozen water in 100 gm. of "frozen" beef muscle have been calculated; these are given below.

TABLE II

Molality of sodium chloride	v (from (5))	v (experimental)	v ($\bar{L}_1 = 0$)
0.1969	0.674	0.674	
1.381	4.68	4.70	4.68
3.138	11.56	11.4	11.8
4.098	16.0	15.5	16.6
5.215	21.9	21.2	22.9

v	1.0	4.7	11.6	16.0	21.9
Free water, *gm*	70.3	15.1	6.6	5.1	4.0

The total amount of unfrozen water in beef muscle (total initial water content 77 per cent) at different temperatures has been determined by Heiss (1933). Values interpolated from his results are given in the second column of Table III. The fourth column is the difference between total (experimental) and free water (calculated) at each temperature and gives the amount of bound water at that temperature.

It will be seen that the values for bound water are of the same order as those for frog's muscle at 0°C. It is not believed that any quantitative conclusion can be drawn from the values in the last column regarding the effect of decrease in temperature and activity of water on the degree of hydration of the muscle proteins. They show, however, that the results from the vapour pressure isotherm and from the freezing-point curve are both in agreement with a low value for the amount of bound water in muscle. A similar conclusion is reached when the results of Moran (private communication) for the freezing-point curves of both frog and beef muscle are compared with the calculated values of v. It should be pointed out that even if the total amount of unfrozen water at −20°C. (determined by Heiss) is considered as bound the value is considerably smaller than those given by Rubner or Thoenes.

TABLE III

Weight of muscle = 100 gm.
Dry weight of muscle = 23 gm.

Temperature	Total water	Free water	Bound water
°C.	gm.	gm.	gm.
−1	77.0	70.3	6.7
−4.7	19.5	15.1	4.4
−11.6	11.4	6.6	4.8
−16.0	9.2	5.1	4.1
−21.9	7.8	4.0	3.8

The System Invariant Point

The temperature at which all the free water in frog's muscle is frozen out can only be estimated very roughly. From the vapour pressure isotherm it can be seen that the equilibrium weight of a muscle is practically constant below $a'' = 0.5$ (down to $a'' = 0.1$). It can be concluded therefore that only a negligible amount of free water is present over this range, *i.e.* the drying-up point of the muscle at 0°C., as regards free water, occurs in the region of $a'' = 0.5$. Substitution of $a'' = 0.5$ in (1) gives the system invariant point as −69°C. This temperature is too low as $\bar{L}_1$ is not negligible at this activity of water. The insolubility of sodium chloride below $a'' = 0.798$ at 0°C. does not allow the calculation of v from (5) for the case of $a'' = 0.5$, but a rough idea of the effect of the heat terms can be obtained by extrapolation.

If values of $(\log a_1'' - \log a_1')/\log a_1''$, obtained from (3) for solutions of sodium chloride down to $a'' = 0.798$, are plotted against log a_1'' an approximately straight line is obtained. From this graph the hypothetical value of a_1, when $a_1'' = 0.5$, is 0.553; this figure substituted in (1) gives $v = 59$. As the drying-up point at 0°C. is not known at all accurately it can only be stated that the system invariant point as regards free water probably lies below −40°C. and above −60°C. It is interesting that Heiss (1933) obtained a value of −62° to −65°C. for the system invariant point from the temperature-time cooling curve of beef muscle. The probable effect of such a low temperature and activity of water on the water of hydration of the proteins is unknown, but the presence of bound water in frog's muscle (at 0°C.) at values of a'' well below 0.5 suggests that even lower temperatures would be required to freeze completely this water of hydration.

SUMMARY

1. The amount of free unfrozen water, *i.e.* water acting as normal solvent, in frog's muscle at temperatures below the initial freezing-point has been calculated from the vapour pressure isotherm of the muscle.

2. Significant amounts of free water are present at −20°C. The total amount of unfrozen water at −20°C. cannot, therefore, be taken as a measure of the bound water in muscle.

3. The calculated values of free water, when compared with experimentally determined values of total unfrozen water, indicate that the amount of bound water in muscle at various temperatures is small.

4. A temperature considerably below −20°C., roughly between −40° and −60°C., is required to freeze completely the free water in muscle.

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THE INFLUENCE OF MINIMAL NARCOTIC DOSES ON THE RESPIRATION OF ERYTHROCYTES

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The investigations of Warburg in 1921 on the action of narcotics on cell respiration led to the formulation of his general theory of narcosis, according to which the action of a narcotic is attributed to its specific ability to occupy the vital surfaces of the cell structures, thereby displacing the adsorbed nutritive substances and important oxidative enzymes (1). The occasional observations of some investigators, describing a stimulating action of narcotics on the metabolic functions of the cell, seem hardly compatible with Warburg's theory; for, "adsorption displacement," as Warburg calls the phenomenon, might bring about a depression, but not a stimulation of cellular metabolism.

In his experiments on the respiration of the isolated spinal cord of the frog under the influence of ethyl alcohol, Winterstein (2) describes a series of cases in which low concentrations of this narcotic produced acceleration, higher concentrations, on the other hand, produced metabolic depression. Similar results were obtained by Garrey (3) in his experiments on the stimulating effect of ethyl alcohol on the CO_2 production of the heart ganglia of *Limulus polyphemus*. Warburg, who made similar investigations on liver cells, avian erythrocytes, and the central nervous system, could find no such acceleration. Narcotic concentrations lower than those which caused metabolic depression were without effect. In explanation of this discrepancy between his results and those of other investigators, Warburg suggested (9) that metabolic acceleration would result upon administering a narcotic to starving cells. The narcotic would then play the rôle of a nutritive substance and as such might be replaced by glucose or peptone with the same stimulating effect. Of course, in the case of cells such as sea urchin eggs, where lipoid-soluble substances serve as a stimulus to further development, as was first demonstrated by Jacques Loeb, an increase in the metabolism of the organism was expected and actually found by him to be the case.

In view of these divergent observations it appeared of special interest to investigate systematically and with modern methods the influence of low concentrations of narcotics on cellular respiration, and to limit ourselves to two narcotics: ethyl alcohol, which can be oxidized and utilized by the cell for nutritive purposes, and ethyl urethan, which is hardly oxidized and certainly not useful as a cellular nutrient. Convenient cells for such investigations were mammalian erythrocytes. It seemed further expedient to extend our investigations to starving erythrocytes as well as to blood cells well supplied with nutritive material.

Method

Human blood was obtained by puncture of the cubital vein; rabbit blood from the auricular vein; horse blood directly from the jugular vein at the slaughter house. Sodium citrate was used to prevent clotting. For the experiments on starving blood cells the citrate blood was centrifuged, the supernatant plasma removed, the erythrocytes suspended in the fivefold volume of physiological saline solution, well stirred, centrifuged, and the supernatant fluid again removed. This process was repeated five times to assure complete removal of all the diffusible nutrients.

In the experiments on nourished erythrocytes the citrate blood was centrifuged but once and the supernatant citrate plasma removed. The sedimented cells were thus still saturated with nutritive material.

5 cc. of the thick erythrocyte suspension were then pipetted into small respiration vessels of the type described by Warburg (4), and 5 cc. of the narcotic to be examined, dissolved in physiological saline solution, added. A vessel containing the erythrocyte suspension plus physiological saline solution, and one with the dissolved narcotic alone, served as controls. The vessels, attached to their manometers, were placed in a constant temperature water bath at 37°C. Air served as the gas medium. The experiments lasted no longer than 2 hours to avoid possible interferences due to bacterial growth.

EXPERIMENTAL

In our search for a concentration of alcohol which would stimulate respiration in erythrocytes it was necessary to start off with inhibiting concentrations. Thus, we found that 4 per cent alcohol caused a depression in O_2 consumption of about 35 per cent. As the concentration of alcohol was gradually lowered this depression became smaller, finally changing into an indifferent behavior when a concentration of 0.5 per cent was reached. Upon lowering the concentration still further we observed a definite increase in O_2 consumption in the range between 0.1 per cent and 0.3 per cent alcohol. To make sure that

TABLE I

Blood	No. of experiments	Concentration of alcohol	Average O_2 consumption in 2 hrs.		Increase or decrease in O_2 consumption
			Without alcohol	With alcohol	
		vol. per cent	*c.mm.*	*c.mm.*	*per cent*
Rabbit	2	4.0	26.9	17.3	−35.7
"	2	2.0	26.9	22.9	−14.9
"	2	1.5	40.1	36.3	−9.5
"	2	0.5	40.1	40.5	+0.1
"	2	0.3	16.8	18.2	+8.3
"	8	0.25	32.5	39.5	+21.5
"	3	0.125	22.7	30.5	+34.3
"	12	0.10	31.3	45.2	+44.5
Human	1	0.5	44.4	40.2	−9.4
"	2	0.25	44.4	57.2	+29.0
"	2	0.10	32.4	46.8	+44.4
Horse	3	0.25	81.5	127.5	+56.4
"	4	0.10	82.4	106.8	+30.0

TABLE II

Blood	No. of experiments	Concentration of urethan in	Average O_2 consumption in 1½ hrs.		Increase or decrease in O_2 consumption
			Without urethan	With urethan	
		per cent	*c.mm.*	*c.mm.*	*per cent*
Human	2	3.0	89.9	52.8	−41.2
"	1	0.02	67.9	72.0	+6.0
"	9	0.015	127.2	118.1	−7.1
"	3	0.005	105.4	94.9	−9.9
Rabbit	1	0.25	28.1	27.4	−2.5
"	1	0.125	27.8	27.0	−2.9
"	2	0.10	28.0	25.0	−10.7
"	1	0.04	84.4	77.9	−7.7
"	2	0.025	84.4	88.0	+4.2
"	3	0.015	39.3	40.5	+3.0
"	1	0.005	26.5	24.6	−7.1

this observation was not based on mere chance some twenty-five experiments were made within this range, both on nourished and starving rabbit erythrocytes. All the results indicated a decided respiratory stimulation, also in the cases of human and horse blood cells. In all three instances starving (washed) erythrocytes reacted the same as nourished blood cells, so that no differentiation of these two categories is made in Table I.

In the instance of ethyl urethan, however, we could find no corroboration of the results obtained with ethyl alcohol, either with starving or with well nourished cells. The values given in Table II fluctuate around the zero mark, indicating an indifference on the part of the erythrocytes towards this narcotic. The general trend, however, points to a slight inhibitory effect. The few positive results shown in the table fall within the limits of error of the manometric method and are therefore hardly of any significance. Occasional controls made with the Barcroft-Warburg differential manometer (4) confirmed the results given in Tables I and II.

DISCUSSION

Our experiments indicate that low concentrations of an easily oxidizable narcotic such as ethyl alcohol increase the O_2 consumption of starving as well as nourished erythrocytes, whereas subinhibitory doses of ethyl urethan remain without effect very likely because this narcotic cannot be utilized by the cell. The fact that even well nourished erythrocytes respond towards low concentrations of alcohol with an increased O_2 consumption can be explained by the observations of Durig and coworkers (5), who showed that upon simultaneous supply of alcohol and carbohydrate in the human body the narcotic is given preference over the carbohydrate and is oxidized first. This also seems plausible for the cell, especially since ethyl alcohol readily penetrates the cell wall of the erythrocyte, and as Fleischmann and Trevani (6) showed, is then oxidatively decomposed into acetaldehyde. While Durig could find no general increase in oxidation in the human organism upon alcohol consumption, recent investigations by Bickel and Kanai (7) show that small quantities of this narcotic stimulate, large quantities on the other hand inhibit oxidative processes in the intermediary metabolism of the rabbit. Similar results were recently obtained by Robertson and Stewart (8), who demonstrated an increased O_2 consumption in brain sections of alcoholized rabbits.

Higher concentrations of either oxidizable or non-oxidizable narcotics bring about respiratory inhibition in cells by more completely displacing from the surfaces of the cell structures not only the adsorbed nutrients, but also the oxidases, so that in the instance of alcohol, this narcotic cannot be burned. Our results can also be fitted into Warburg's "adsorption displacement" theory of narcosis by assuming that low narcotic concentrations do not sufficiently displace the adsorbed nutrients and enzymes, thus making an easily oxidizable narcotic readily accessible to the cell, whereas non-oxidizable narcotics remain indifferent.

SUMMARY

Low concentrations of ethyl alcohol stimulate the respiration of mammalian erythrocytes *in vitro*.

Low concentrations of ethyl urethan remain without effect on, or tend slightly towards depressing the respiration of mammalian erythrocytes *in vitro*.

It is suggested that this may be due to the oxidizable nature of alcohol, and the non-oxidizable nature of urethan, properties which come into evidence only when these narcotics are present in such low concentrations that the threshold of inhibition (narcosis) has not been reached.

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THE ULTRAVIOLET ABSORPTION SPECTRUM OF PEPSIN

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I

In connection with measurements of the inactivation (destruction) of dissolved crystalline pepsin by ultraviolet light, determinations were made of the absorption spectrum of preparations of pure crystalline pepsin. From these data, the molecular extinction coefficient curve may be deduced. The pepsin used was prepared by Dr. John H. Northrop; through his kindness sufficient amounts of several preparations were made available (*cf.* Northrop, 1929–30*a*, *b*).

The crystalline pepsin in known dilutions in a suitable buffer solvent was put into 2 cm. glass photometer tubes or into adjustable micro Baly tubes with quartz end-plates. Corresponding control tubes were filled with the same dilution of the solvent solution. The tubes were placed in the paths of twin beams of light in a quartz sector photometer. The light source was a tungsten steel spark, 3 mm. gap, heated with a gas flame, and operated at 6600 volts from a transformer taking 17 amperes at 110 volts on the primary winding.

The transmission spectra were recorded on panchromatic photographic plates, using a large quartz spectrograph (Judd-Lewis, 1919, 1922; *cf.* Gates, 1930–31). The ratio of intensities incident on the two tubes was varied by means of the photometer sector vanes, over a wide range of intensities. When the length of liquid traversed by the light is kept constant the extinction coefficient is calculated from Beer's law. The logarithm of the ratio (I/I_0) is equal to log 1 − log D; log D is obtained from the scale readings on the vanes of the photometer. The molecular extinction coefficients were calculated for the points at which bands of equal density appeared on the spectrograms and then plotted against the corresponding wave-lengths to give the absorption curve.

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* This paper is one of several in which results of work completed by Dr. Frederick L. Gates before his death, June 17, 1933, are reported. The manuscripts have been prepared by Professor W. J. Crozier and Dr. R. H. Oster.

II

Tests were made on a preparation of carefully purified pepsin received from Dr. Northrop in 75 per cent glycerine, containing 1.80 mg. of protein nitrogen per ml., and on samples dissolved in M/100 HCl to make up dilutions of 1/10, 1/50, 1/100. By the quinhydrone electrode the 1 in 50 dilution showed a pH of 2.54; for the other dilutions this was not determined. For these solutions corresponding dilutions of M/100 HCl containing glycerine in the same proportion were used in the control tubes. From the spectrograms obtained with these samples between wave-lengths 2166 and 3130 Å.u., and from the calculated values of the molecular extinction coefficients, the absorption curve for pure pepsin was plotted (Fig. 1). On the basis of a molecular weight of 36,000 (Northrop, 1929–30*b*, p. 771), and the percentage of protein nitrogen of 15.15 (Northrop, 1929–30*a*, p. 747), the molecular extinction coefficient is calculated from the expression

$$\epsilon = \frac{\text{Log } D}{d \cdot M \cdot \text{dilution}}$$

where d is the thickness of solution and M is the molarity, in this case 0.00033 M; $M \times$ dilution is of course identical with C in the formula as usually written.

III

Slightly different values were obtained at high dilutions (1/125, 1/400) in some cases and with less highly purified preparations, but these irregularities were probably due either to pH differences or to uncertainty in regard to the concentration of the enzyme. In all preparations the location of the maxima and minima on the absorption curve coincided.

Other tests showed that even slight differences in the pH had a decided effect on the absorption of ultraviolet energy as indicated by the rate of inactivation of the enzyme. Northrop (1933–34) has shown

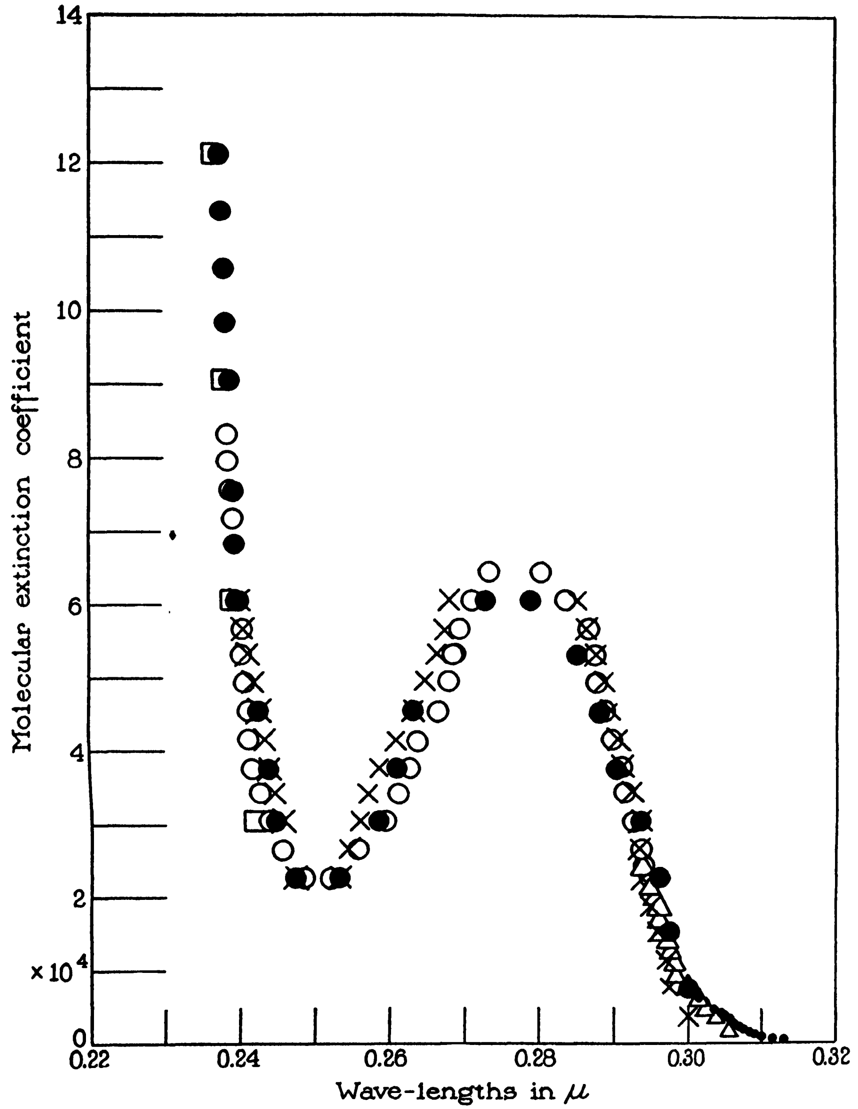

Fig. 1. The absorption curve of carefully purified crystalline pepsin (1.80 mg. of protein nitrogen per ml.) in 75 per cent glycerine dissolved in M/100 HCl to give the following dilutions: Clear circles and solid circles represent points obtained with two solutions of pepsin diluted 1 in 50, pH 2.54, and tested in 2 cm. photometer tubes; small dots, undiluted pepsin solution tested in 1 cm. tubes; crosses, diluted to 1 in 50, pH 2.54, and tested in 4 cm. tubes; triangles, diluted to 1 in 10 and tested in 2 cm. tubes; squares, diluted to 1 in 100 and tested in 2 cm. tubes. The range of intensities on the photometer sector scales was spaced at intervals of $\log_{10} D = 0.1$, between 0.1 and 1.6, to cover the complete range plotted.

that the rate of inactivation depends on the pH of the solution, and this has also been indicated by Collier and Wasteneys (1932) and by Pincussen and Uehara (1928).

IV

As shown in Fig. 1 the absorption approaches a maximum in the region below 2400 Å.u.; a lower peak of absorption occurs at 2750–2800 Å.u.; minimum absorption is near 2500 Å.u. and in the region of wave-lengths longer than 3000 Å.u.

Kubowitz and Haas (1933) determined the absorption curve of urease in the ultraviolet, and measured the decrease in activity of a solution of jack-bean urease (Sumner, 1926) when irradiated with ultraviolet light of measured intensities at wave-lengths between 196 mμ and 366 mμ. They computed from these data the relative absorption spectrum of urease. Since the molecular weight of urease was not known, close comparison of their curve with that found for pepsin is not possible. However, the locations of maximum and minimum of absorption seem to agree rather closely. This is in accordance with the similarities implied in the protein character of the two enzymes, and in the agreement of their crystalline forms, as pointed out by Northrop (1929–30*a*).

The slight but definite changes in the slope of the curves (humps) at 2560–2650 Å.u., at 2930 Å.u., and at 3000 Å.u. may be significant as indicating a difference between the absorption spectrum of pepsin and that of such a substance as tyrosine. These humps may also indicate certain fine characteristics in the absorption of ultraviolet energy associated with enzyme activity, of the type found by Lavin, Northrop, and Taylor (1933) in the absorption spectrum of pepsin at low temperatures.

SUMMARY

The ultraviolet absorption spectrum of Northrop's pure crystalline pepsin has been determined. The curve of calculated molecular extinction coefficients is given. There is noted a general resemblance of the absorption curve for pepsin to that for urease and tyrosine; the absorption band is maximum at 2750–2800 Å.u., minimum near 2500. A slight hump on either side of the peak of the extinction curve may be significant.

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STIMULATION OF FUNDULUS BY HYDROCHLORIC AND FATTY ACIDS IN FRESH WATER, AND BY FATTY ACIDS, MINERAL ACIDS, AND THE SODIUM SALTS OF MINERAL ACIDS IN SEA WATER

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INTRODUCTION

Stimulation of aquatic animals by substances dissolved in the medium will be interpretable when the rôles played both by the environment and by the receptive mechanism are understood. Stimulating forces originating in the environment are effective only when the receptive mechanism is so adjusted that an interaction between environment and receptors is possible. In any one animal the range of receptive processes is strictly limited and it is impossible to change it significantly by experimental procedure without causing "abnormal" reception. Similarly any marked change in the environment is equally disastrous to normal reception in the majority of animals. However, there are a few animals which can live equally well in quite different media. Of these the killifish *Fundulus heteroclitus* is a good example, since it can live in fresh, brackish, or salt water without showing abnormal behavior. If stimulation by the same substance in different media should result in different responses a correlation between the specific environments and the receptive mechanism would be indicated; but if no difference in response appeared, it would be suggestive of an independence of reception upon the change of general environment. This animal has therefore been used to test the stimulating effectiveness of certain acids and salts in fresh and salt water. Hydrochloric and five normal aliphatic acids (acetic, propionic, butyric, valeric, and caproic) were tested in fresh water, while the same acids plus sulfuric and nitric, as well as the sodium salts of the mineral acids, were tested in salt water.[1]

The experimental procedure in all cases was the same as that described for the sunfish (Allison, 1931–32), except that tap water was used instead of spring water for the fresh water tests, and that the rate of flow for the salt water tests was 250 cc. instead of 100 cc. per minute.

When fresh or salt water flows over *Fundulus*[2] under constant experimental conditions the rate of mouth movements and opercular movements remains fairly constant. Occasionally there may be movements of the fins or body and variations in the respiratory movements, but such behavior only temporarily interferes with the tests. When an acid solution replaces fresh or salt water the fish responds by closure of the mouth and opercula. When inorganic salts are used, the response is more often a gaping of the mouth or gulping, followed by closure. The response is usually very definite and easily recognized. The time between turning on the solution and the response is measured and designated the reaction time. During the night and other periods when not being used, the fish are returned to their individual aquaria where feeding occurs. The animals may be used repeatedly for several weeks (8 to 12) and continue to give the same reaction times (±5 per cent) for the same solutions.

The reaction time is a measure of the response to stimulation. For the acids it is correlated with the concentration of the hydrogen ion[3] and for the salts with the normal concentration. In the case of the fatty acids another factor appears to play a rôle in stimulation, namely, the field of force around the non-polar group in the molecule. This factor increases as the number of CH_2 groups increases,

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[1] The experiments with salt water were done at the Mt. Desert Island Biological Laboratory during the summers of 1932 and 1933. Preliminary reports of the results were published in the *Bull. Mt. Desert Island Biol. Lab.*, 1933, 27; 1934, 33.

[2] In any group of *Fundulus* there are always some individuals which cannot be used for this type of experiment, because they move about incessantly. The majority, however, quickly become acclimated to the experimental dish and remain quiet for hours unless stimulated.

[3] The pH of the solutions both in fresh and salt water was measured by the quinhydrone electrode and calculated from the equation:

$$\mathrm{pH} = \frac{-E + 0.4529 + 0.00002t}{0.000019832T}$$

Check determinations by the hydrogen electrode showed variations from the quinhydrone values which were no larger than the errors of measurement. Hydrogen ion concentrations were then calculated from Sørensen's equation:

$$\mathrm{pH} = \log \frac{1}{C_H}$$

and may be indirectly measured by the hydrogen ion concentration. The intensity of stimulation by each acid, as measured by the reaction time, appears to be some function of the activity of the hydrogen ion. There are several ways of correlating these two variables, but they are not all equally suggestive of an interpretation. In choosing a method of representation there are certain points to be noted. First, the reaction time must be corrected to give a value which is clearly related to the stimulus. As measured by the stop-watch the reaction time includes a certain minimum time required by (1) the passage of the afferent nervous impulse to the central nervous system; (2) internuncial coordination; (3) the passage of the efferent impulse to the effectors; and (4) the processes of muscular contraction which close the mouth and opercula. As long as the intensity and frequency of stimulation are low enough to avoid all secondary effects, such as adaptation, narcosis, toxicity, and the like, that time is constant. It should therefore be subtracted from that observed reaction time, since it is not correlated with the stimulus. Furthermore a certain time is lost before the solution comes into contact with the receptors, and its magnitude depends upon the rate of flow of solution over the fish and upon the volume of the container. This should also be subtracted from the reaction time. For the experiments in fresh water with a flow of 100 cc. per minute the combined correction factor was 4 seconds, as determined by the maximum rate of reaction at different rates of flow. For the salt water tests with flows of 250 cc. per minute, only 2 seconds were needed to correct the observed reaction time. Secondly, it is desirable to use *rate of reaction* instead of reaction time in seconds, since rate, considered as a function of some variable, is easier to interpret than measured time. Finally the data are so expressed that when one variable is plotted against the other a linear relationship appears.

Experiments in Fresh Water

Using *Fundulus* in fresh water, hydrochloric, acetic, propionic, butyric, valeric, and caproic acids were tested at several concentrations each. As with the catfish *Schilbeodes* (Cole and Allison, 1931–32), a single *Fundulus* gives as reliable results as several individuals averaged together, and makes unnecessary the correction for individual variations in thresholds. Table I summarizes all the data secured from a single fish. In Fig. 1 the logarithm of rate of reaction is plotted against logarithm of (H^+), and the lines are drawn from the following equation[4] which relates rate of reaction with (H^+):

$$\log \frac{100}{RT - 4} = 0.665\,[1 - 0.5^{(1 + 0.7n)}] \log ((H^+) \times 10^6) + 0.56 \qquad (1)$$

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[4] The equation was derived from the line drawn through the HCl points, so that it would represent fair agreement with the other points and approach a limiting value when $n = 6$.

TABLE I

Reaction Times of Fundulus to Acids in Fresh Water

Temperature: 18 ± 0.2°C. Rate of flow: 100 cc. per minute. $n = 10$. The values in Column 3 were calculated from equation (1).

pH	Observed $RT - 4$	Calculated $RT - 4$	Probable error of $RT - 4$*
Hydrochloric			
	sec.	*sec.*	
3.02	2.72	2.81	0.1060
3.17	3.30	3.09	0.1776
3.44	3.80	3.88	0.2312
4.00	6.00	5.95	0.5471
4.68	9.30	10.02	0.7106
Acetic			
3.74	2.69	2.49	0.1996
3.90	3.79	2.97	0.2473
4.10	4.42	3.68	0.2893
4.33	5.67	4.69	0.3934
4.64	7.61	6.52	0.6294
5.10	14.78	10.61	0.8922
Propionic			
3.75	2.20	1.69	0.0903
3.92	2.73	2.08	0.1326
4.16	3.30	2.81	0.1269
4.65	4.70	5.16	0.2015
5.06	8.04	8.58	0.4850
5.21	13.65	10.33	0.5386
Butyric			
4.10	2.23	2.11	0.1286
4.21	2.65	2.44	0.1100
4.38	2.74	3.08	0.1410
4.88	6.49	6.05	0.4641
4.98	5.40	6.93	0.2087
5.29	9.88	10.54	0.6926
Valeric			
4.20	2.31	2.13	0.1714
4.34	2.99	2.60	0.1553
4.79	4.03	4.92	0.1438
4.93	6.10	6.02	0.7219
5.35	7.61	10.93	0.5104
Caproic			
4.52	2.94	3.16	0.1714
4.85	4.58	5.12	0.1375

* P.E. = $\pm 0.8453 \dfrac{\Sigma(+v)}{n\sqrt{n-1}}$

where n = number of CH_2 groups in the acid molecule. (For hydrochloric acid $n = 0$.) For each acid the equation may be written in the simplified form:

$$RT - 4 = \frac{1}{K(H^+)^b}, \tag{2}$$

where K and b are constants which fix the position of each line. There are definite experimental limits within which these equations describe the process of stimulation of *Fundulus* by acids in fresh water. These limits are defined by the plot. Since the lines all intersect where reaction time = 27.5 seconds and the $(H^+) = 1 \times 10^{-6}$ N, it is obvious that extrapolation of the equations below these points would have no experimental justification. The common intersection further shows that stimulation by any of the acids may be correlated solely with the potential of the hydrogen ion at the lowest (H^+) value. Although the lines drawn from equation (1) do not fit some of the plotted points

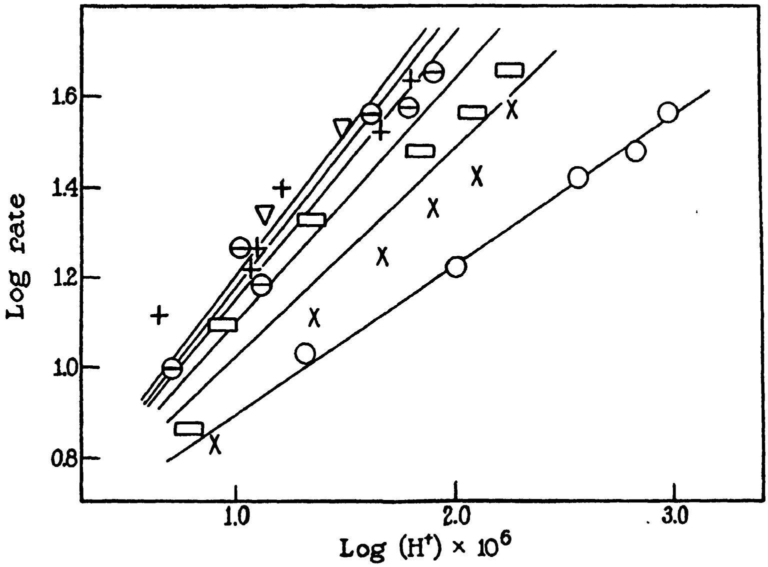

FIG. 1. Log of rate of reaction $\left(\text{where rate} = \frac{100}{RT - 4}\right)$ plotted against log $(H^+) \times 10^6$ for stimulation of *Fundulus* in fresh water by the following acids: HCl (○), acetic (×), propionic (▭), butyric (⊖), valeric (+), caproic (▽). (See text for further description.)

very well, the following interpretation would still be justified even though slightly different lines or no lines at all were drawn.

Stimulation by HCl is correlated with the effective hydrogen ion concentration. Stimulation by the fatty acids, as their concentration increases, is correlated with two factors: the effective hydrogen ion concentration, and the potential of the non-polar group in the molecules. The latter potential increases by a fixed amount as each CH_2 group is added to the molecule beginning with acetic acid. However, not all of this added energy is available for stimulation, since as the number of CH_2 groups increases the stimulating effect increases by smaller and smaller amounts, and gradually approaches a maximum value, as shown by equation (1), where the limiting slope approaches 0.665. This situation differs from stimulation by alcohols, where each CH_2 group increases effectiveness by a constant amount (Cole and Allison, 1930–31).

The results correspond qualitatively with those obtained on the sunfish (Allison, 1931–32; Cole and Allison, 1932–33 *a*). Both *Eupomotis* and *Fundulus* react in the same way to mineral and fatty acids. In each fish stimulation by the mineral acids may be correlated with the effective hydrogen ion concentration, and stimulation by the fatty acids with length of the carbon chain. However, there are quantitative differences. The sunfish has a higher threshold for stimulation by hydrochloric acid than *Fundulus* and the mathematical statements describing the relationship between (H^+) and rate of reaction also differ for the two fish. Stimulation by the fatty acids in *Eupomotis* is correlated primarily with the field of force around the non-polar group, which is not true for *Fundulus*. Furthermore in the sunfish a limiting stimulating value determined by the number of CH_2 groups in the molecule is not reached so quickly as in *Fundulus*. The difference in behavior must be due to differences in the receptive mechanism. Although the equilibrium is shifted in both cases by a change in (H^+), a shift of stimulating proportions is reached at a lower (H^+) in *Fundulus* than in *Eupomotis*, which means that the dynamic equilibrium between receptors and environment is different in the two fish. The simplest assumption to make is that the chemical natures of the receptors differ. Such a difference would also account for the different behavior of the two fish towards stimulation by the fatty acids.

In *Fundulus* the interface might become saturated with fatty acids lower in the series than in the sunfish, so that addition of more CH_2 groups to the molecule produces no further increase in stimulation, while in *Eupomotis* saturation occurs only with the higher acids in the series, allowing increased stimulation as more CH_2 groups are added. In both fish the concentration of fatty acids at the receptor surface, as determined by the length of the carbon chain and by the composition of the receptor surface, would increase the activity of the hydrogen ion at the interface beyond that of the medium as a whole. If it is further assumed that the only rôle of the non-polar group in the fatty acids is to concentrate the acids at the receptor interface, then equal rates of reaction in one fish would be produced by equal hydrogen ion concentrations at the surface, regardless of the acid used, even though the hydrogen ion concentrations of the media as a whole were quite different for the different acid solutions. However, since intensity of stimulation is always measured by a change in hydrogen ion concentration of the media, stimulation by the fatty acids must be correlated wholly or in part with a force which is not measured in terms of primary valence.

Experiments in Sea Water

(*a*) *Mineral Acids*

With *Fundulus* adapted to sea water the stimulating efficiencies of HCl, H_2SO_4, and HNO_3 were tested at several concentrations. Ten observations on each of two fish were made at each concentration. The two sets of data were so similar that they were averaged (see Table II). In Fig. 2 the logarithm of the rate is plotted against the logarithm of the (H^+), revealing a linear relationship over the experimental range. Per cent variation[5] is essentially constant, indicating no change in the mechanism of the stimulation process. The three mineral acids evidently enter into the reaction in equivalent amounts. Stimulation by these acids must therefore be correlated with the so called chemical forces of primary valence which can be measured by the hydrogen ion concentration. The line in Fig. 2 is much like the

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[5] Per cent variation $= \frac{100 \text{ P.E.}}{RT - 2}$. The average per cent variation for the mineral acids in sea water = 6.7. (*Cf.* Allison, 1931–32, and Cole and Allison, 1932–33 *a*.)

TABLE II

Reaction Times of Fundulus to Mineral Acids in Sea Water

Temperature: 17.7 ± 0.2°C. Flow: 250 cc. per minute. $n = 20$, 10 on each of two fish.

pH	$RT - 2$	Probable error*	pH	$RT - 2$	Probable error	pH	$RT - 2$	Probable error
Hydrochloric acid			Sulfuric acid			Nitric acid		
	sec.			*sec.*			*sec.*	
2.70	1.66	0.1148	2.68	1.76	0.1241	2.70	1.46	0.0712
3.12	2.36	0.1556	2.89	2.06	0.1226	2.90	2.01	0.0942
3.18	3.24	0.2487	3.24	2.23	0.0976	3.27	2.64	0.1272
3.34	2.67	0.1336	3.53	3.24	0.2594	3.61	2.97	0.1083
3.52	2.65	0.1883	3.85	3.14	0.1754	3.81	3.23	0.1827
3.60	2.77	0.1480	4.16	4.22	0.2924	3.95	3.82	0.2479
3.79	3.30	0.2510	4.46	5.76	0.3714	4.14	4.29	0.2188
3.93	3.79	0.2842	4.53	4.61	0.4097	4.49	5.16	0.3787
4.00	3.57	0.2960	4.72	5.15	0.3039	4.94	6.16	0.4286
4.08	4.71	0.2941	5.08	7.93	0.5787	5.19	9.13	0.5059
4.34	5.01	0.3464	5.10	6.29	0.4689	5.28	8.79	0.5528
4.42	7.23	0.4912	5.22	11.66	0.8513	5.47	9.32	0.6046
4.49	5.95	0.5550	5.41	10.24	0.4450	5.50	11.47	0.8760
4.77	5.57	0.5651	5.50	10.26	0.5549	5.73	10.23	0.4987
4.85	7.37	0.5758	5.60	11.85	0.6900	5.75	11.67	0.6933
5.00	5.88	0.5919	5.87	14.24	0.8995	5.85	14.23	0.9199
5.18	8.81	0.6488						
5.23	10.16	0.7912						
5.25	12.60	1.1939						
5.70	14.47	1.4681						
5.80	11.50	0.8056						
5.80	14.08	1.0389						

$$* \text{P.E.} = \pm 0.8453 \frac{\Sigma(+v)}{n\sqrt{n-1}}$$

HCl line in Fig. 1, and can be described by the same equation (Equation 2) with slightly different constants, as follows:

$$\text{for HCl in fresh water: } RT - 4 = \frac{1}{3.59(\text{H}^+)^{0.3325}} \qquad (3)$$

$$\text{for HCl, H}_2\text{SO}_4\text{, and HNO}_3\text{ in sea water: } RT - 2 = \frac{1}{3.716(\text{H}^+)^{0.232}} \qquad (4)$$

Both equations are subject to about the same experimental limits. Such close agreement indicates that a change from fresh to sea water does not alter the type of reaction at the receptor interface when mineral acids are added to the environment of *Fundulus*. By increasing the (H^+) of either fresh or sea water with mineral acids, the established equilibrium existing between receptors and environment is so shifted that the same response occurs.

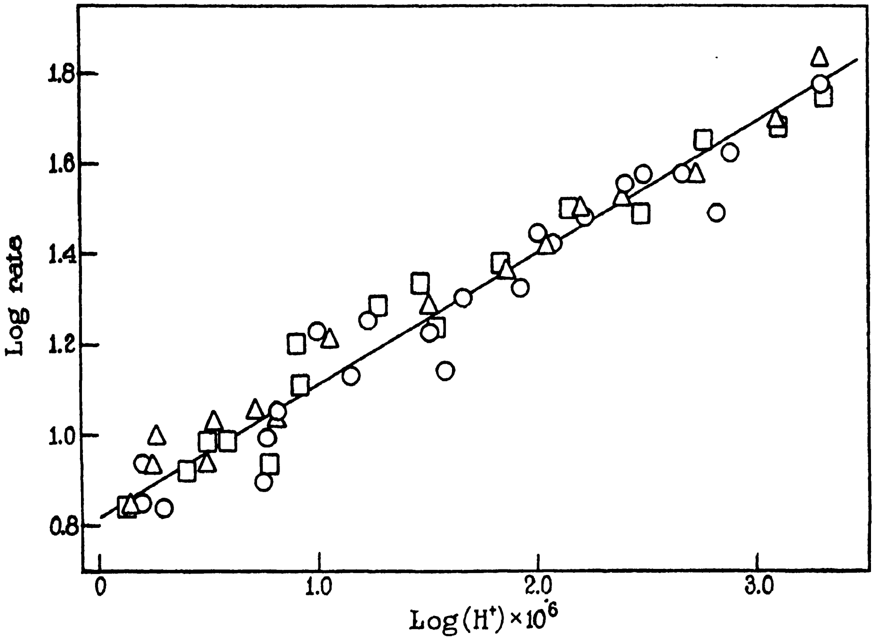

FIG. 2. Log of rate of reaction $\left(\text{where rate} = \frac{100}{RT - 2}\right)$ plotted against log ($(H^+) \times 10^6$) for stimulation of *Fundulus* in sea water by HCl (○), H_2SO_4 (□), and HNO_3 (△). (See text for further description.)

A year previously qualitative data were secured for stimulation by HCl and by the fatty acids (acetic, propionic, butyric, valeric, and caproic) in sea water, and are presented in Fig. 3. Although the threshold for stimulation by HCl was slightly lower, the slope of the line is the same as that for HCl in Fig. 2, showing excellent agreement with the later tests. Here again evidence exists that the fatty acids are more effective than the mineral acids because of the presence of CH_2 groups in the molecules. Although there are not sufficient data to justify a quantitative treatment, the difference in spread of the lines for fatty acids in Fig. 1 and Fig. 3 is considered significant, and not as due to experimental or other systematic errors. In fact, a difference might be expected because over the whole experimental range it was necessary to use higher concentrations of fatty acids in sea water than in fresh water in order to produce the same pH. For example, 0.0004 N propionic acid in sea water changed the pH from 8.2 to 7.4, while in fresh water it changed the pH from 8.0 to 5.2. A lower concentration of free fatty acid and a higher concentration of the salts of the fatty acids therefore exist in the sea water solutions than in the fresh water solutions. Furthermore, there is a greater change in CO_2 pressure in the sea water solutions than in fresh water, since sea water is buffered mostly by bicarbonates. Since little difference was found between the stimulating efficiencies of the mineral acids in fresh and in sea water, a change in CO_2 pressure is probably not the reason for the different effects of the fatty acids in the two environments. However,

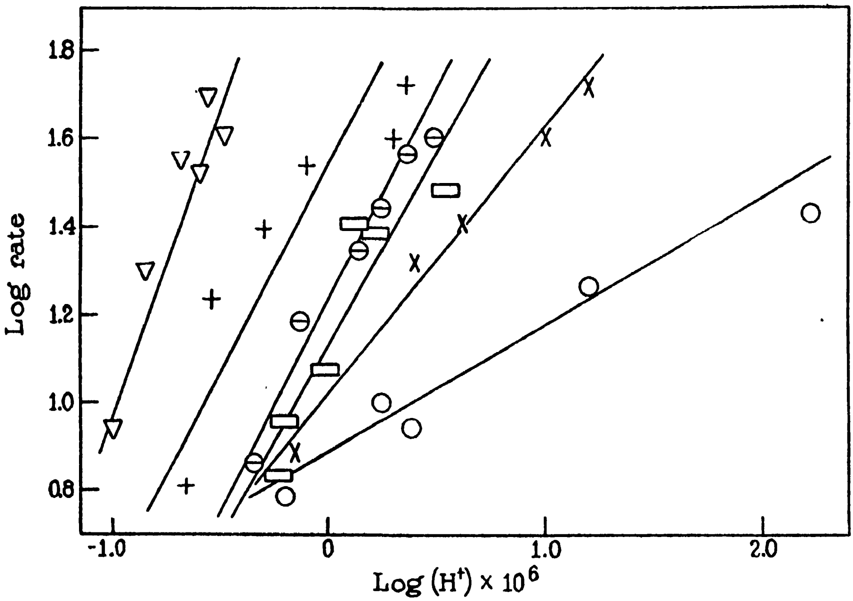

FIG. 3. Log of rate of reaction $\left(\text{where rate} = \frac{100}{RT - 2}\right)$ plotted against log $((H^+) \times 10^6)$ for stimulation of *Fundulus* by the following acids: HCl (○), acetic (×), propionic (□), butyric (⊖), valeric (+), caproic (▽). (See text for further description.)

part of this difference must be explained by the formation of higher concentrations of the fatty acid anion in sea water than in fresh water. The tendency of this anion to alter the complex ionic equilibrium at or near the receptor interface in sea water has been recognized in the interpretation of experiments with the barnacle (Cole, 1931–32; Cole and Allison, 1932–33b).

TABLE III

Reaction Times of Fundulus to Inorganic Salts in Sea Water

Temperature: 17.7 ± 0.2°C. Flow: 250 cc. per minute. $n = 20$, 10 on each of two fish. Concentration normal = normal concentration of salt added to sea water.

Concentration normal	$RT - 2$	Probable error	Concentration normal	$RT - 2$	Probable error	Concentration normal	$RT - 2$	Probable error
Sodium nitrate			Sodium chloride			Sodium sulfate		
	sec.			*sec.*			*sec.*	
0.08	19.74	1.0459	0.08	16.91	1.0825	0.08	17.46	1.2627
0.085	18.12	1.5865	0.085	16.53	1.3863	0.09	13.61	1.0326
0.09	17.94	1.3477	0.09	14.60	1.0163	0.10	10.45	0.4977
0.095	13.91	1.3316	0.095	16.77	1.1956	0.11	9.49	0.4458
0.10	11.62	0.4090	0.10	10.88	0.5479	0.12	9.52	0.7352
0.105	12.69	0.9380	0.105	12.81	0.8540	0.13	7.87	0.4901
0.11	10.57	0.4498	0.11	13.28	0.8194	0.14	5.92	0.2916
0.115	8.55	0.6945	0.115	8.43	0.4430	0.16	5.03	0.2918
0.12	8.70	0.3578	0.12	8.93	0.5916	0.18	5.23	0.2989
0.125	9.97	0.8812	0.125	8.91	0.5947			
0.13	8.23	0.5231	0.13	9.29	1.0025			
0.135	9.61	0.9159	0.135	9.80	0.6529			
0.14	6.07	0.3076	0.14	8.72	0.6412			
0.145	6.15	0.5837	0.145	8.93	0.3677			
0.15	6.56	0.4235	0.15	7.82	0.8446			
0.155	4.93	0.6720	0.155	7.83	0.6110			
0.16	4.95	0.3117	0.16	6.45	0.6895			
0.165	5.46	0.5710	0.165	5.72	0.4269			
0.17	4.85	0.2594	0.17	6.80	0.6601			
0.175	5.35	0.5563	0.175	6.71	0.7402			
0.18	4.80	0.3158	0.18	4.69	0.3372			
			0.19	5.16	0.5062			

(*b*) *Inorganic Salts*

Tests were also made with the sodium salts of HCl, H_2SO_4, and HNO_3; the data are collected and summarized in the same way as for the acids (see Table III and Fig. 4). The pH of the salt solutions was the same as that of sea water. The responses of the fish to a change in salt concentration of the sea water were not as regular as they were to a change in (H^+), and often consisted of a gaping of the mouth or gulping followed by closure. Sometimes no easily recognizable response occurred when the salt solutions were used, especially at the lower concentrations. Therefore a few "no reactions" were interspersed with the obvious reactions. The former were not included in the analysis of the data. This difference in behavior of the fish towards stimulation by the salts and acids may indicate a difference in the mechanism of the stimulation processes. The average percentage variation for the salts was 7.5, which is slightly higher than for the acids, but all of the points lie within two parallel lines defining the scatter. The average line may be represented by the following equation within the experimental limits:

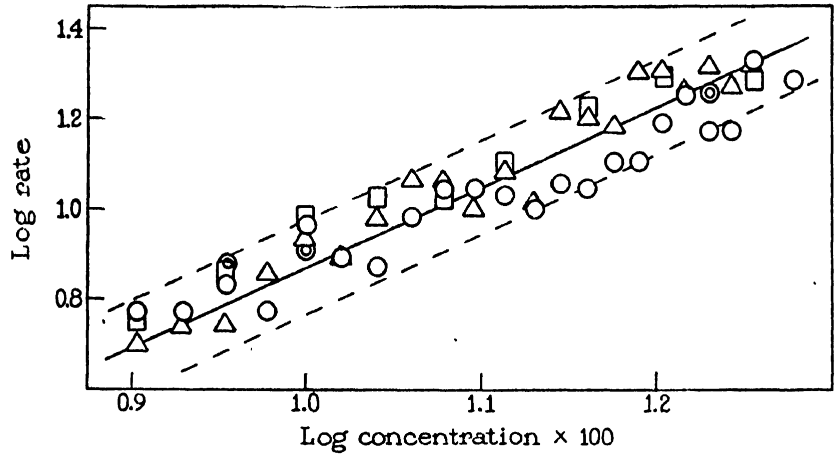

FIG. 4. Log rate of reaction $\left(\text{where rate} = \frac{100}{RT - 2}\right)$ plotted against log concentration × 100 for stimulation of *Fundulus* by NaCl (○), Na_2SO_4 (□), and $NaNO_3$ (△) in sea water. The three points marked by two concentric circles (◎) are the averages of twenty observations on each of the three salts by a different observer using a different individual *Fundulus* at a different rate of flow (100 cc. per minute). To correct these reaction times 4 seconds were subtracted instead of 2.

$$RT - 2 = \frac{1}{4.498\ C^{1.78}}, \qquad (5)$$

where C represents equivalent concentrations of the salts added to sea water. Check tests for each of the salts indicated by concentric circles in Fig. 4 were made by another observer on a single fish adapted to a different experimental set-up with a lower rate of flow (100 cc. per minute). There is no apparent break in the relationship stated by equation (5), since the per cent variation is independent of the change in concentration.

Although much higher concentrations of the anions were used in the salt solutions than in the acid solutions, there is no evident effect which can be correlated with difference in the polarity of these ions. Sodium chloride, sodium sulfate, and sodium nitrate enter into the reaction in equivalent amounts, so that surface forces which might alter such a stoichiometric relationship play no rôle. Stimulation by these salts as well as by the mineral acids can therefore be correlated with the forces of primary valence. However, the stimulating efficiency of the sodium salts measured by threshold concentrations is much less than for the acids. The greater threshold concentration of the salts might be expected since the fish is adapted to a relatively high concentration of sodium ions in sea water. The efficiency of the reaction in stimulation by salts and by acids may also be measured by the slopes of the lines in Fig. 4 and Fig. 2. The greater the slope the greater the efficiency of the reaction over the experimental range. The concentration exponent in equation (5), which is the slope of the line, is 1.78 while the exponent in equation (3) for the mineral acids is 0.292. Therefore after the respective threshold concentrations have been reached the sodium salts are more effective as stimulating agents than the mineral acids.

SUMMARY

1. *Fundulus heteroclitus* was found to be a reliable experimental animal for studies on chemical stimulation in either fresh or sea water.

2. The response of *Fundulus* to hydrochloric, acetic, propionic, butyric, valeric, and caproic acids was determined in fresh water, while the same acids plus sulfuric and nitric, as well as the sodium salts of the mineral acids, were tested in sea water.

3. Stimulation of *Fundulus* by hydrochloric acid in fresh water is correlated with the effective hydrogen ion concentration. Stimula-

tion by the *n*-aliphatic acids in the same environment is correlated with two factors, the effective hydrogen ion concentration and the potential of the non-polar group in the molecule. However, as the number of CH_2 groups increases the stimulating effect increases by smaller and smaller amounts, approaching a maximum value.

4. Stimulation of *Fundulus* by hydrochloric, sulfuric, and nitric acids in sea water is correlated with the forces of primary valence which in turn are correlated with the change in hydrogen ion concentration of the sea water. The *n*-aliphatic acids increase in stimulating efficiency in sea water as the length of the carbon chain increases, but a limiting value is not reached as soon as in fresh water.

5. Only a slight difference in stimulation by hydrochloric acid is found in sea water and in fresh water. However, there is a significant difference in stimulation by the fatty acids in fresh and in sea water, which is partly explained by the different buffering capacities of the two media. It is to be noted that in the same environment two different fish, *Fundulus* and *Eupomotis*, give different results, while the same fish (*Fundulus*) in two different environments responds similarly to mineral acids but differently to fatty acids. These results illustrate that stimulation is a function of the interaction between environment and receptors, and that each is important in determining the response.

6. Stimulation by sodium chloride, nitrate, and sulfate is correlated with equivalent concentrations of the salts added to sea water, or with the forces of primary valence. Although the threshold for stimulation by the salts is considerably higher than for the acids, the efficiency of stimulation by the salts is greater.

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MANOMETRIC MEASUREMENTS OF PHOTOSYNTHESIS IN THE MARINE ALGA GIGARTINA

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I

We undertook the following study of photosynthesis in *Gigartina* partly in the hope of generalizing some of our conclusions drawn from work with *Chlorella*, but the results seem interesting in several other respects as well. The rather primitive methods used heretofore in measuring photosynthesis in marine algae do not permit much control of external conditions. From the existing knowledge of photosynthesis in fresh water aquatics and terrestrial plants, it is clear that results cannot be interpreted unless the degree of saturation with light and carbon dioxide is known. These essentials have never, as far as we are aware, been established in the experiments with marine algae, yet the results have been made the basis of important generalizations about the photosynthesis and ecology of these organisms. Several investigators have concluded that the Florideae are enabled, by the activity of their characteristic red pigment, to grow successfully at great depths, and in general where the color and intensity of the light are unfavorable to the development of green and brown algae.

In this paper, we cannot undertake to discuss adequately the question of the photosynthetic activity of phycoerythrin, or the distribution of the Florideae, nor do we think that a study of a single species, such as we have carried out, would provide proper basis for such a discussion. We mention these problems only because our experiments indicate the kind of information which will have to be collected before such questions can be discussed profitably.

The shortcomings in the technique of experimenters on photosynthesis in marine algae are illustrated by the work of Ehrke (1931). He, like Kniep (1914), Harder (1915), and Montfort (1929), kept his material under so called "natural" conditions, because he was attacking an ecological problem. To measure photosynthesis, he enclosed samples of algae in glass vessels of special design, and of about 1 liter capacity. They were filled to the neck with sea water, stoppered tightly, and exposed to light for several hours, either in the laboratory, or at various depths in the sea. Photosynthesis was determined by titrating the oxygen content of samples of sea water taken before and after exposure. Ehrke gives the dry weight of the material for each

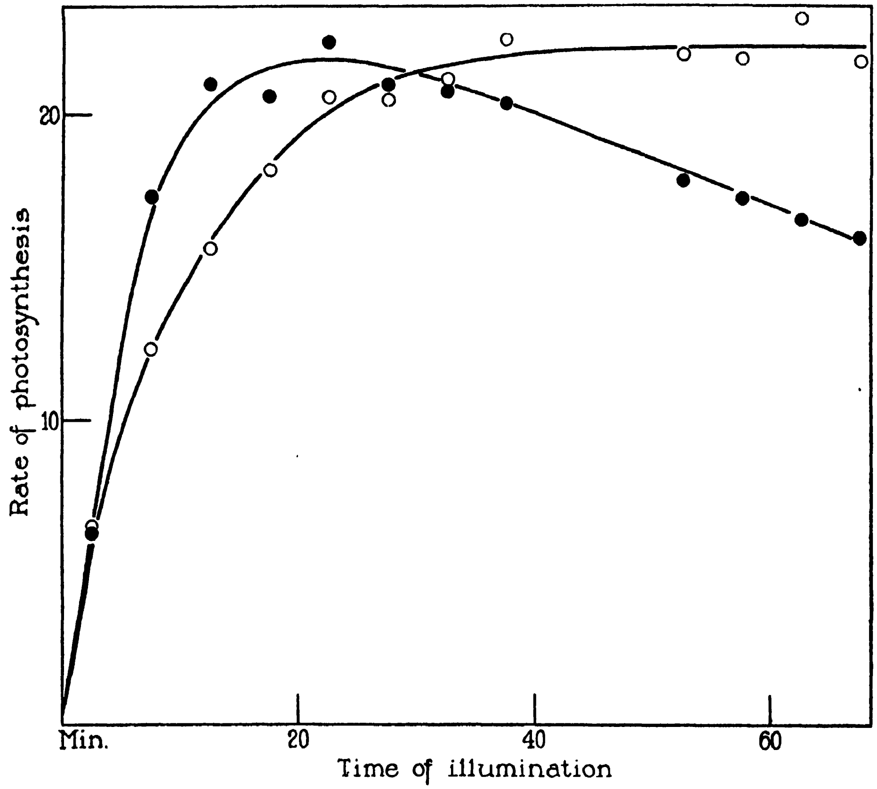

FIG. 1. *Gigartina* photosynthesis in ordinary sea water (solid circles), and in ordinary sea water saturated with 5 per cent CO_2 in air (open circles). Temperature = 15°C. Rate of photosynthesis is in mm. of Brodie per 5 minutes for 2.1 cm.^{2} of material.

experiment. Using Harder's (1915) table of dry weights, we have estimated that Ehrke used about 50 gm. of fresh material in about a liter of sea water, for a typical experiment.

In Fig. 1 we have plotted relative rates of photosynthesis against time of illumination. The curve with solid circles represents measurements with a piece of *Gigartina* weighing less than 150 mg., suspended in 8 cc. of ordinary sea water. Illumination began at zero on the time scale. Photosynthesis rises to a maximum in about 15 minutes, and soon afterwards enters an almost linear decline. This shows that when *Gigartina* and sea water are used in these proportions, only the measurements of the second 15 minutes of illumination are approximately constant. In Ehrke's experiments more material was used in proportion to the amount of sea water, and he determined only the average rate for 3 hours or more. The curve with solid circles in Fig. 1 shows that this may be something quite different from the rate of photosynthesis under truly "natural" conditions, when the algae are bathed continuously in fresh sea water.

The decline in rate is not due to irreversible injury from the experimental conditions, but to a steady fall in the carbon dioxide concentration, because of its removal in photosynthesis. This is well shown by the curve with open circles in Fig. 1. Here the same amount of material was suspended in the same quantity of ordinary sea water, this time saturated with 5 per cent carbon dioxide in air. Under these conditions the rate shows no sign of dropping for 2 hours or longer.

II

Methods

Photosynthesis was measured with Barcroft-Warburg manometers and rectangular vessels, of the type illustrated by Emerson (1929, p. 614). We shall discuss in detail the control of carbon dioxide concentration in sea water, and mention briefly the provisions made in this investigation for the other external factors.

Carbon Dioxide Concentration.—Ordinary sea water contains a small amount of dissolved carbon dioxide, a much larger amount of bicarbonate, and some carbonate. If the dissolved carbon dioxide is used for photosynthesis, some of the bicarbonate will decompose to form carbonate, and more carbon dioxide. But the concentration of the carbonate-bicarbonate system is too low to maintain a constant concentration of carbon dioxide in our experiments, and we have already explained that the rate of photosynthesis falls unless some additional supply of carbon dioxide is provided. The curve with solid circles in Fig. 1 shows that rate measurements may be made in the second 15 minute period after the beginning of illumination, while the rate stays approximately constant. Because of the ready availability and constant composition of ordinary sea water, this may often be a desirable practice. Whenever we have used this technique, we have calculated the oxygen production, or photosynthesis, on the assumption that the pressure changes are entirely due to evolved oxygen, and that the partial pressure of carbon dioxide remains constant for this short period of time. This is a rather crude assumption, because of the poor buffering effect of the bicarbonate in the sea water. It tends to make the calculated oxygen production, and hence the rate of photosynthesis, lower than the true value.

A constant rate of pressure change can be maintained for several hours by suspending material in ordinary sea water saturated with 5 per cent carbon dioxide in air. The curve with open circles in Fig. 1 shows measurements of photosynthesis in this medium. It shows no tendency to decline, even at the end of 70 minutes, and the same is true of much longer periods. To calculate gas exchange from pressure changes in such a system, we may regard it as analogous to Warburg's Ringer solution saturated with 5 per cent carbon dioxide (*cf.* Warburg, 1924). Two vessels containing unequal volumes of fluid are required for each determination. Carbon dioxide consumption and oxygen production, and hence the photosynthetic quotient CO_2/O_2, are calculated from the pressure changes in the two vessels. Pressure changes in a single vessel can be used as a measure of relative rate of photosynthesis when the quotient is unknown. The curve with open circles in Fig. 1 was made in this way. Material in ordinary sea water saturated with 5 per cent carbon dioxide attains a steady rate of photosynthesis more slowly than material in other media, as is illustrated by Fig. 1. For this reason we have preferred to use artificial sea water without carbonate or bicarbonate, saturated with 5 per cent carbon dioxide, for measuring the photosynthetic quotient. The experiments made in ordinary sea water saturated with 5 per cent carbon dioxide serve as a useful check on those made under more artificial conditions. Either ordinary or artificial sea water saturated with 5 per cent carbon dioxide has at 15° a carbon dioxide concentration of very nearly 0.002 molar. But artificial sea water saturated with the gas mixture may be objectionable because it is considerably more acid than other media we have used. The ordinary sea water at Pacific Grove had a pH of 7.6. When saturated with 5 per cent carbon dioxide, the pH fell to 6.6, while the value for artificial sea water without carbonates, and saturated with 5 per cent carbon dioxide, was about 5.3.[1] In spite of the acidity, photosynthesis in this medium continues at a constant rate for some time.

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[1] We are indebted to Dr. K. V. Thimann for making the pH determinations for us, with a glass electrode.

In the majority of our experiments, carbon dioxide was provided by adding to artificial sea water larger amounts of carbonate and bicarbonate than are present in ordinary sea water. The advantages of carbonate mixtures for studying photosynthesis have been brought out by Warburg's work with *Chlorella* (1919). They exert a buffering effect on the carbon dioxide concentration, and maintain it nearly constant so that photosynthesis can be measured by oxygen production. Warburg has been widely criticized for his use of carbonate mixtures, which constitute a rather unphysiological medium for *Chlorella*. Whether or not these criticisms are justified,[2] they have little or no bearing on the use of carbonate mixtures for marine algae, because ordinary sea water is already a dilute carbonate-bicarbonate mixture. The normal pH of ocean water is said by Harvey (1928) to be about 8.1. In strengthening the concentration and varying the composition of the carbonate mixture, we have confined ourselves to a pH range of 7.6 to 8.6, a slight variation from the normal.

Warburg's solutions were of 0.1 molar concentration, and were well buffered. Such concentrated mixtures cannot be prepared in sea water without precipitating calcium and magnesium carbonates. Artificial sea water could be prepared with less calcium and magnesium, and the salinity made up by using more sodium and potassium chloride. Such a medium would probably be uninjurious to marine algae for short periods, and would permit the use of more concentrated carbonate mixtures, resulting in a a valuable improvement in their buffering capacities. We have made no experiments in this direction.

Our artificial sea water was prepared according to the specifications of E. J. Allen (1914, p. 421), except that the chlorides were not titrated, and ordinary distilled water from a tin-lined still was used. The following stock solutions were mixed as indicated:

Stock Solution	*Amount of stock per 100 cc. sea water cc.*
28.3 gm. NaCl in 500 cc. H_2O	50.0
KCl, molar solution	1.0
$CaCl_2$, " "	1.1
$MgCl_2$, " "	2.6
$MgSO_4$, " "	2.9

After mixing, distilled water was added to make 84 cc., and then 16 cc. of carbonate-bicarbonate mixture were added. The stock solutions must be mixed and diluted before adding the carbonate mixture, to avoid precipitation of carbonates.

Buch and several collaborators (1932) made an exhaustive study of carbonate equilibria in sea water, and compiled tables which facilitate the calculation of the carbon dioxide concentration and acidity of our various carbonate mixtures in artificial sea water. We shall indicate the form in which their data are given, and the essential steps in calculating carbon dioxide concentration.

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[2] Since preparing this paper, we have made experiments which show that at least at high concentrations of carbon dioxide, the rate of photosynthesis in *Chlorella* is quite independent of pH between 4.6 and 8.6. This objection to Warburg's procedure appears to be invalid.

When carbon dioxide physically dissolved in water is in equilibrium with the carbon dioxide in the air, the concentration $[CO_2]$ in the water, expressed in mols per liter, is related to the partial pressure p_{CO_2} of carbon dioxide in the air, expressed in atmospheres, by the equation

$$[CO_2] = c \cdot p_{CO_2}.$$

Here c is a constant called the molar solubility of carbon dioxide, whose value depends on the temperature and the quantity of dissolved solids. Buch's report includes a table of molar solubilities of carbon dioxide in sodium chloride solutions for temperatures from −2° to 30°C., and for salt contents or salinities (S) from zero to 40 °/₀₀. The symbol °/₀₀ denotes parts per thousand by weight. The solubilities in sea water do not differ appreciably from those in sodium chloride solutions of corresponding temperature and salinity. (The "salinity" of sea water refers to the total salt content.) Therefore we can use these values in calculating the concentration of carbon dioxide in sea water if we know its partial pressure in the air. The concentrations in ordinary and artificial sea water saturated with 5 per cent carbon dioxide were calculated in this way.

The dissociation of the dissolved carbon dioxide may take place through either of two intermediate steps:

$$(a) \quad CO_2 + H_2O = H_2CO_3 = H^+ + HCO_3^-,$$

$$(b) \quad H_2O = H^+ + OH^-, \; CO_2 + OH^- = HCO_3^-.$$

Either of these pairs of reactions leads, by elimination of the intermediate step, to the following mass action expression for the equilibrium concentrations:

$$\frac{[H^+]\,[HCO_3^-]}{[CO_2]\,[H_2O]} = K.$$

Since the concentration of water molecules is constant for a water sample of given salinity and temperature, its value may be incorporated in the constant. The bicarbonate ion dissociates further:

$$HCO_3^- = H^+ + CO_3^=.$$

We have, then, using Buch's notation for the constants:

$$\frac{[H^+]\,[HCO_3^-]}{[CO_2]} = K_{c_1},$$

$$\frac{[H^+]\,[CO_3^=]}{[HCO_3^-]} = K'_2.$$

These may be solved for the carbon dioxide concentration or the hydrogen ion concentration:

$$[CO_2] = \frac{K_2'}{K_{c1}} \cdot \frac{[HCO_3^-]^2}{[CO_3^=]}, \tag{1}$$

$$[H^+] = K_2' \cdot \frac{[HCO_3^-]}{[CO_3^=]}. \tag{2}$$

The values of K_{c_1} and K'_2 vary with both the temperature and the salinity of the water. According to the theory of Debye and Hückel (Clark, Chapter 25), as well as to experiment, these constants are functions of the ionic strength μ of the solution, which is one-half the sum of the products obtained by multiplying the concentration of each ion in the solution by its valence. All dissolved salts are considered to be completely ionized.

Natural sea water, though varying widely in salinity, maintains a very constant relative composition, so that the concentration of a single constituent completely determines the composition. Thus, sea water is commonly characterized by its chlorine content. The ionic strength, salinity, and chlorine content of natural sea water are directly proportional to one another, as follows:

$$\mu = 0.020 \times S \text{ ‰} = 0.036 \times Cl \text{ ‰}.$$

Buch's tables give values of pK_{c_1} and pK'_2, defined respectively as $-\log_{10} K_{c_1}$ and $-\log_{10} K'_2$, tabulated as functions of chlorine content, for temperatures from 0° to 30°C. and for chlorine contents up to 22 ‰. The ionic strength is the true determining factor, and only in the case of natural sea water can this factor be stated in terms of chlorine content. The importance of using these values for the constants, determined in sea water, in place of the usual values for the constants as determined in extremely dilute solutions is realized when we note that, for example, at 20°C. in extremely dilute solutions K'_2 has the limiting value 3.5×10^{-11}, while in ordinary sea water (Cl = 19 ‰) its value is 1.7×10^{-9}, about fifty times as great.

Dr. MacInnes has derived a value of K'_2 for sea water of the same salinity by extrapolation of a curve published by MacInnes and Belcher (1933), which differs greatly from the value obtained from Buch's figures. But these authors made their determinations in more dilute solutions than Buch, whose determinations were made in sea water, and a considerable extrapolation was required. Buch and others have pointed out that such constants are a different function of μ in dilute and in concentrated solutions, so we have preferred to use Buch's values, believing them to be the best obtainable at present. While future work on carbonate equilibria may lead to different values for the constants, and hence modify our absolute carbon dioxide concentrations, the relative concentrations in the various mixtures will probably remain unchanged, as Warburg pointed out in connection with his own mixtures (1919, p. 317). Warburg used constants different from ours to calculate carbon dioxide concentrations in his mixtures, so his figures cannot be compared directly with ours, but in each case the shape of the curves and the conclusions to be drawn from them are not likely to be modified by changes in the constants.[3]

To solve equations (1) and (2), the concentrations of carbonate and bicarbonate ions must be known. The potassium carbonate and bicarbonate in our artificial sea water may be regarded as completely ionized, so we may set the initial ion concentrations equal to the concentrations of their respective salts. Here, as elsewhere, we refer to concentration per liter instead of per kilo. This involves only a negligible error.

Table I shows the initial carbon dioxide concentrations and pH values of our mixtures of carbonate and bicarbonate in artificial sea water at 15°, calculated from equations (1) and (2). We also give the concentrations of carbon dioxide in mixtures A and E for 4°, because these mixtures were used in experiments at different temperatures. The pH changes only slightly with temperature.

TABLE I

Initial Conditions in Artificial Sea Water with Various Carbonate Mixtures

$$[HCO_3^-] = 0.016\beta$$
$$[CO_3^=] = 0.016\,(1 - \beta)$$

Mixture	β	15°			4°
		pH	p_{CO_2} in atmospheres × 10^4	$[CO_2]$	$[CO_2]$
				mols × *10^5/l.*	*mols* × *10^5/l.*
A	5/8	8.6	7	2.7	2.4
B	3/4	8.3	15	5.8	
C	13/16	8.2	23	9.1	
D	7/8	7.9	41	16.0	
E	15/16	7.6	93	36.0	33.0

Two factors operate to alter the initial carbon dioxide concentrations of our mixtures. The algal material withdraws carbon dioxide for photosynthesis, and carbon dioxide is exchanged between the mixture and the gas space until the two are in equilibrium. The latter is a negligible factor in our experiments, because the gas space is always small, and the partial pressures of carbon dioxide in equilibrium with our various mixtures are never much above its partial pressure in the atmosphere. The amount of carbon dioxide used in photosynthesis is large compared with that which may leave the mixture as gas, and it is important to know how much may be used without greatly changing the concentration. Mixture E, with the highest concentration, has the lowest buffering capacity. The removal of 50 c.mm. of carbon dioxide from 8 cc. of mixture causes a fall of about 20 per cent in the concentration. The removal of an equal amount from mixture A causes a fall of only about 5 per cent in concentration.

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[3] Later work of Buch has resulted in a revision of his values of pK'_2. The revised value for K'_2 at 20°C. and Cl 19 $^0/_{00}$ is 9.8×10^{-10}. This would reduce our calculated values for carbon dioxide and hydrogen ion concentrations about 40 per cent. *Cf.* Buch, K., *J. conseil internat. l'exploration de la mer*, 1933, **8**, 309.

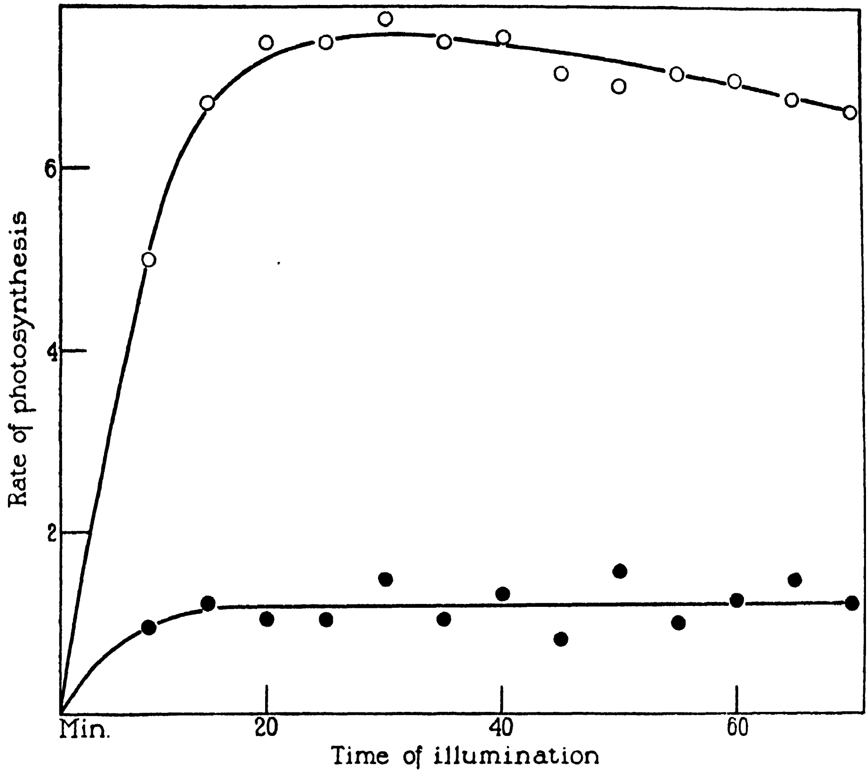

FIG. 2. *Gigartina* photosynthesis in artificial sea water to which has been added a mixture of potassium carbonate and bicarbonate. The solid circles are for a mixture giving a low carbon dioxide concentration, the open circles for a mixture giving a high concentration. Temperature = 15°C. Rate of photosynthesis is in mm.^{3} O_2 per 5 minutes for 2.1 cm.^{2} of material.

The course of photosynthesis in *Gigartina* in two mixtures corresponding approximately to A and E is shown in Fig. 2. In the lower concentration (solid circles), the rate remains at the same level throughout the experiment. In the higher concentration, after the initial rise, the rate remains fairly constant for about 20 minutes, and then begins to show a decline, because of falling carbon dioxide concentration. In making experiments in these carbonate mixtures, we have confined our measurements to the time interval when the rate is nearly constant, the second 20 minute period after the beginning of illumination.

It is evident that better buffer mixtures could have been prepared by keeping the carbonate constant, instead of decreasing it, as the bicarbonate was increased. At the time we did our experimental work, information on carbonate equilibria in sea water was not available, and we had expected to use a method of calculating carbon dioxide concentration similar to Warburg's. This .requires that the concentration of the cation, potassium in our case, be kept constant, while the carbonate and bicarbonate are varied. The method of calculation outlined above, using Buch's tables, does not depend on the constancy of the cation concentration, so there is more latitude than we allowed ourselves in preparing our mixtures.

Lighting.—Illumination was provided by a row of internally frosted 60 watt lamps, placed as close to one another as possible, and about 8 cm. from the bottoms of the vessels containing the algal material. The number of lamps was always two greater than the number of vessels, not counting the control, which contained no material.

To vary the intensity, Wratten neutral filters were attached to the bottoms of the vessels with rubber bands. The manufacturer's transmission figures for this series of filters have been found to be correct within 3 per cent for incandescent light. To insure that no light entered the vessels except through the filters, the sides and tops were covered with jackets of copper foil.

Temperature.—Because of the known sensitivity of marine algae to higher temperatures, we conducted our measurements at temperatures of 16° or lower. The surface water at Pacific Grove is seldom if ever warmer than 16°C. The vessels were shaken in a large water thermostat, the temperature of which was maintained constant to less than 0.05°C.

III

Material

We made trial experiments with *Iridaea*, *Nitophyllum*, and *Gigartina*. A species of *Gigartina*, identified for us by Professor N. L. Gardner, of the University of California, as *G. harveyana*, was selected as most suitable for our purposes. Juvenile fronds are to be found in moderate quantity throughout the summer at Pacific Grove. They grow in a narrow zone about a foot below mean lower low water. The young fronds are smooth, thin, free from epiphytic algae, and of a fairly uniform red color. They were collected at low tide, and kept in a tank of running sea water in the laboratory. We found they could be kept in good condition for 2 weeks or longer, but after this time they showed a somewhat reduced rate of photosynthesis. The experiments described in this paper were made with material kept less than 7 days in the laboratory.

Pieces of thallus to fit in the manometer vessels were punched out with a 16.5 mm. cork borer, or with an oval stainless steel punch which cut an area almost exactly twice that of the cork borer. We have used 2.1 cm^2. and 4.2 cm^2. for their respective areas.

The manometer vessels were filled with measured volumes of fluid, and then the pieces of thallus were introduced, one piece to each vessel, immediately after being cut. The change in fluid volume made by introducing the piece of thallus with its adhering water was estimated by weighing a series of pieces cut with the oval punch from fronds differing as much as possible in size and thickness. The weights varied from 270 to 330 mg. In any one experiment the variation was surely much smaller, because care was taken to cut all pieces for a given experiment from one frond, and as close together as possible. The pieces were assumed to have a density of 1, and a uniform addition to the fluid volume was made in all cases, 300 c.mm. for the oval pieces, and 150 c.mm. for the circular pieces.

The respiration of marine algae is generally supposed to be small. In *Gigartina* it is about 1/30 of the maximum photosynthesis at 15°, so it is an insignificant correction under optimum conditions. But at low intensities or carbon dioxide concentrations it becomes an appreciable proportion of the photosynthesis. In general we have dispensed with measuring it, and have made a small arbitrary correction for it. Nothing is changed in our conclusions if the correction is omitted, but the lower points in some of the figures fall more nearly on the curves when it is included.

Photosynthesis is expressed in cubic millimeters of evolved oxygen, calculated from change in pressure. In making the calculations, the absorption coefficient α given in Landolt-Börnstein for oxygen in 0.5 molar sodium chloride has been used. This value may not be quite correct for sea water, but since α_{O_2} changes only slightly with salt concentration, and constitutes only a small factor in the calculations, it may safely be used for our experiments.

IV

Description and Discussion of Experiments

One of the most characteristic features of the photosynthetic process in *Chlorella*, and probably in green plants in general, is the behavior of the temperature coefficient, Q_{10}. At high light intensities, the coefficient runs from 2 to 6, depending on the range studied. Reduction in the rate of photosynthesis by lowering the light intensity causes the coefficient to fall to nearly unity, whereas the same reduction in rate brought about by lowering the carbon dioxide concentration, keeping the light intensity high, does not greatly alter the coefficient. If photosynthesis in the Florideae involves the same reaction mechanism as in the green plants, then the Florideae may be expected to react in the same way to temperature changes under these conditions.

The choice of light intensities and carbon dioxide concentrations for the experiments at different temperatures must be determined by measurement of photosynthesis as a function of these two factors.

Fig. 3 shows a plot of rate of photosynthesis at 15°C., against light intensity in arbitrary units. The highest intensity is the direct light from a row of closely spaced 60 watt lamps, about 8 cm. from the algal material. Table II gives complete data for the experiment. The coordinates of the points in Fig. 3 are in Columns 1 and 7. The curve shows that the material is nearly saturated by the full light intensity.

Although the shape of this curve is somewhat different from Warburg's intensity curve for *Chlorella* (1925, p. 388), this does not necessarily indicate any difference in the two photosynthetic processes. Warburg used thin cell suspensions, which reduced the intensity not more than 10 per cent as the light passed through them. Our sections of *Gigartina* may have reduced the intensity of the incident light as much as 90 per cent as it passed through them. This means that for Warburg's curve, all cells will reach saturation at about the same incident intensity, because they are all nearly equally illuminated, whereas for our curve, the cells farthest from the light will reach saturation only after the incident light has far exceeded the intensity necessary to saturate the cells first receiving the light. Consequently we should expect our curve to show a much more gradual approach to saturation, and this is actually the case.

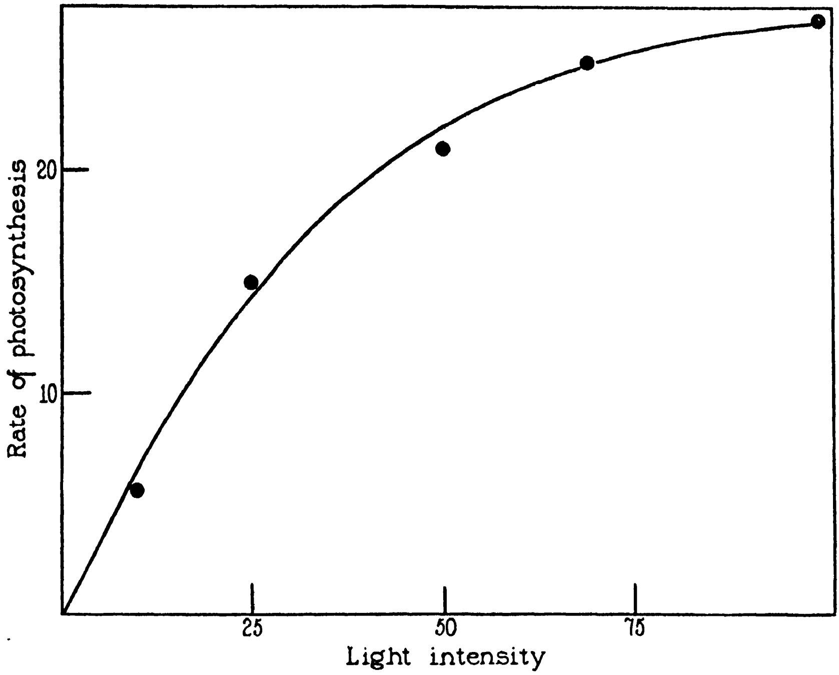

FIG. 3. Rate of *Gigartina* photosynthesis as a function of light intensity. The unit of intensity is arbitrary. Temperature = 15°C. Rate of photosynthesis is in mm.^{3} O_2 per 15 minutes per cm.^{2} of material.

TABLE II

Photosynthesis at Different Light Intensities. Detailed Data for Fig. 3

Area of algal material in each vessel = 2.1 cm.^{2} Material suspended in ordinary sea water in equilibrium with air. Light from six 60 watt lamps close together, 8 cm. below vessels.

Temperature = 15.0°C.

Relative intensity (per cent transmission of filter)	Volume of gas space	Volume of fluid, including material	Vessel constants K_{O_2}	Δh change of pressure in 15 min.	x_{O_2} volume of oxygen evolved in 15 min. = $\Delta h\ K_{O_2}$	x_{O_2} with uniform correction for respiration, 1 mm.^{3} in 15 min.
	cc.	*cc.*		*mm.*	*mm.*^{3}	*mm.*^{3}
10	4.16	7.15	0.41	11.1	4.6	5.6
25	4.13	7.15	0.41	33.6	14	15
50	5.26	8.15	0.52	37.6	20	21
75	5.08	8.15	0.51	47.2	24	25
100	5.33	8.15	0.53	48.9	26	27

We should mention here that the pieces of thallus never turn over while they are being shaken in the manometer vessels. During an entire experiment they remain with the same side facing the full intensity of the light, and the other side in deep shadow.

Montfort (1930) quotes Wurmser as stating that *Rhodymenia* shows a diminution in photosynthesis when exposed to intense light. He attributes this to the presence of phycoerythrin, because *Ulva* does not show a similar diminution. *Gigartina* may be exposed to intense light for several hours, and still show an undiminished rate of photosynthesis, provided the supply of carbon dioxide remains adequate, so we think the phenomenon observed by Wurmser can hardly be due to the presence of phycoerythrin.

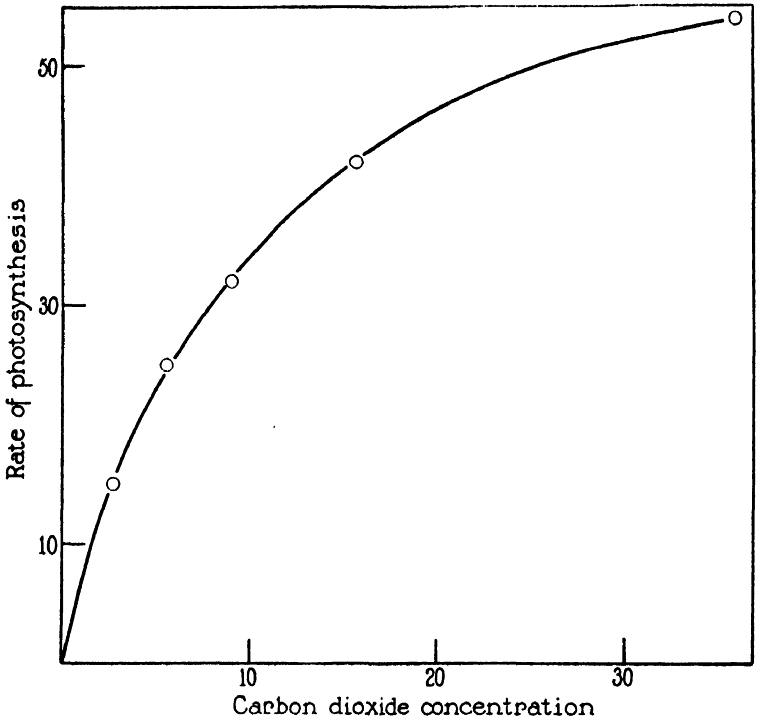

FIG. 4. Rate of *Gigartina* photosynthesis as a function of carbon dioxide concentration. Photosynthesis is in mm.^{3} O_2 per hour per cm.^{2} of material, and CO_2 concentration in mols × 10^5. High light intensity; temperature = 15°C.

Fig. 4 shows the rate of photosynthesis at 15°C., plotted against carbon dioxide concentration. Table III gives complete data, and the coordinates of the points are in Columns 2 and 10. The highest point on the curve may be a little low, because of the poor buffering capacity of mixture E, which we discussed in the section on methods. The curve is similar to those for *Chlorella* photosynthesis obtained by Warburg (1919, p. 254) and by Emerson and Arnold (1932, p. 409). The rate is a linear function of the log of the carbon dioxide concentration.

TABLE III

Photosynthesis at Different CO_2 Concentrations. Detailed Data for Fig. 4

Area of algal material in each vessel = 2.1 cm.^{2}

Material suspended in artificial sea water with carbonate mixtures described in Table I.

Q_{O_2} is the rate of photosynthesis, expressed as volume of O_2 evolved per hour per cm.^{2} of material, corrected for respiration.

Temperature = 15.0°C.

Carbonate mixture	Mols. CO_2 per liter $\times 10^5$	Volume of gas space	Volume of fluid including material	Vessel constant K_{O_2}	$\Delta h'$, change of pressure in 20 min. in dark	x'_{O_2}, respiration per hr. $= -3\Delta h' K_{O_2}$	Δh, change of pressure in 30 min. in light	x_{O_2}, oxygen produced in light per hr. $= 2\Delta h K_{O_2}$	Q_{O_2} $\left(\frac{x_{O_2} + x'_{O_2}}{2.1}\right)$
		cc.	*cc.*		*mm.*	*mm.*^{3}	*mm.*	*mm.*^{3}	*mm.*^{3}
A	2.7	4.13	7.15	0.41	−2.3	2.8	33.3	27.3	15
B	5.8	4.16	7.15	0.41	−3.2	3.9	61.0	50.0	25
C	9.1	5.34	7.15	0.52	−2.3	3.6	61.0	63.5	32
D	15.8	5.08	8.15	0.51	−2.7	4.1	82.1	83.7	42
E	36.0	5.33	8.15	0.53	−1.7	2.7	103.5	110	54

We have investigated the effect of temperature on rate of photosynthesis in mixture E at full light intensity, and with the 10 per cent filter; and in mixture A at full light intensity. The results are shown in Fig. 5, rate being plotted directly against temperature in degrees Centigrade. The curve with solid circles is for high intensity and high carbon dioxide concentration, the open circles for low carbon dioxide concentration and high intensity; and the crosses for low intensity and high carbon dioxide concentration. Table IV gives complete data, and the coordinates of the points are in Columns 1 and 8.

We have found that exposure of our material to temperatures between 4° and 16° caused no injury, and that within this range all temperature effects were reversible.

Fig. 5 indicates that the mechanism of photosynthesis in *Gigartina* must be fundamentally like that in *Chlorella*. A Blackman reaction, with a high temperature coefficient, governs the rate at high intensities, and a photochemical reaction, with a coefficient of about 1 governs the rate at low intensities. It seems evident that at low carbon dioxide concentrations the rate is determined by a chemical reaction, rather than by the diffusion of carbon dioxide, because the value of 4.4 for Q_{10}, calculated for the increase in rate from 4° to 8°, is too high to represent a diffusion process. Van den Honert (1930), using *Hormidium*, failed to find a coefficient much higher than 1 at low concentrations of carbon dioxide, and interpreted this as showing that

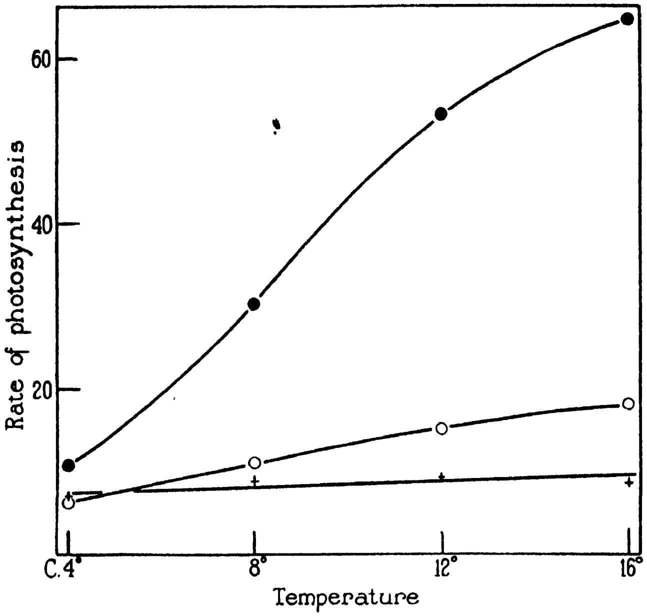

FIG. 5. Rate of *Gigartina* photosynthesis plotted against temperature in degrees Centigrade. Solid circles are for high intensity and high CO_2 concentration, open circles for high intensity and low CO_2 concentration, and crosses for low intensity and high CO_2 concentration. Photosynthesis is in mm.^{3} O_2 per hour per cm.^{2} of material.

the rate of photosynthesis was governed by the diffusion of carbon dioxide. From his curves, this appears likely; but we believe that they represent limiting conditions outside the cell due to his peculiar experimental method. In the case of *Gigartina*, owing to the thickness of the material it seems probable that the lower coefficient found at low carbon dioxide concentration, compared to the coefficient for high carbon dioxide concentration and high light intensity, is due in part to diffusion.

TABLE IV

Temperature Coefficients under Various Conditions. Detailed Data for Fig. 5

Light from four 60 watt lamps close together, 8 cm. below vessels. For low intensity experiment, vessels were covered with 10 per cent transmission filters.

Q_{O_2} is the rate of photosynthesis, expressed as volume of O_2 evolved per hour per cm.^{2} of material, corrected for respiration.

Conditions of experiment	Temperature	Volume of gas space	Volume of fluid including material	Vessel constant K_{O_2}	Δh change of pressure in 20 min.	Volume of O_2 per hr. per cm.^{2} material	Respiration in dark per hr. per cm.^{2} material	Q_{O_2}
	°C.	cc.	cc.		mm.	mm.^{3}	mm.^{3}	mm.^{3}
I. High I, high [CO_2]. 2.1 cm.^{2} material suspended in artificial sea water with carbonate mixture E	4	5.33	8.15	0.56	12.4	9.9	0.7	10.6
	8	4.13	7.15	0.43	47.2	29	1.2	30
	12	5.26	8.15	0.53	66.7	51	1.6	53
	16	5.33	8.15	0.53	82.6	63	2.1	65
II. High I, low [CO_2]. 4.2 cm.^{2} material suspended in artificial sea water with carbonate mixture A	4	4.93	8.3	0.52	14.5	5.4	0.7	6.1
	8	4.01	7.3	0.42	32.7	9.8	1.2	11.0
	12	5.19	7.3	0.52	35.8	13.3	1.6	14.9
	16	4.93	8.3	0.49	45.0	15.8	2.1	17.9
III. Low I, high [CO_2]. 4.2 cm.^{2} material suspended in ordinary sea water	4	3.98	7.3	0.42	21.0	6.3	0.7	7.0
	8	4.01	7.3	0.42	24.9	7.5	1.2	8.7
	12	3.98	7.3	0.40	26.4	7.5	1.6	9.1
	16	6.18	7.3	0.60	14.8	6.3	2.1	8.4

The effect of temperature on photosynthesis by marine algae has been investigated by Kniep (1914), Harder (1915), and Ehrke (1931). Kniep suggested that the ability of marine forms to develop successfully in the polar seas might depend on a greater fall in respiration than in photosynthesis as the temperature drops. Harder is also of this opinion, but his experiments give it meager support. His figures show that the value of Q_{10}, for *Fucus* respiration, is about 1.2 between 4° and 16°. Over this range, photosynthesis usually shows a Q_{10} of 3 to 4, with a marked increase at the lower temperatures. This is certainly the case with *Gigartina.* Q_{10} for photosynthesis is 1.7 from 12° to 16°, and 13.5 from 4° to 8°.

Ehrke claims that his curves substantiate Kniep's view. They show various photosynthetic maxima at low temperatures. Sometimes there is a declining rate with rising temperature, and another maximum at a higher temperature. Because of the extreme unlikelihood of such temperature functions, we must regard Ehrke's curves as the result of a combination of poor experimental technique and injury at higher temperatures, unless they can be substantiated by reliable methods.

We realize that external conditions may be such that somewhat more oxygen is produced at low than at high temperatures, because at sufficiently low light intensity photosynthesis practically does not change with temperature. This is a perfectly general characteristic, and should not be represented as a special adaptation to enable marine algae to inhabit the polar seas. It is also far from adequate to explain Harder's statement that the oxygen production of *Fucus* increases more than twenty times as the temperature falls from 17° to 0°, especially in view of Harder's own respiration measurements on *Fucus*, which show, as mentioned above, a Q_{10} of only 1.2 between 4° and 16°.

To compare the effect of temperature on *Gigartina* photosynthesis quantitatively with similar measurements for other forms, we have plotted our results according to the Arrhenius equation. We do this only to bring out similarities and differences which are otherwise not obvious, and we do not wish to imply anything concerning the mechanism of the temperature effect.

For our purposes the Arrhenius equation may be written:

$$\frac{R_2}{R_1} = e^{\frac{\mu}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)}$$

where R_1 and R_2 are rates of reaction at absolute temperatures T_1 and T_2, R is the gas constant, e the base of natural logarithms, and μ

a constant whose value expresses the effect of temperature on the reaction. The work of Crozier and his collaborators has shown that μ is rarely constant for biological processes over their entire temperature range, but may be nearly constant for short intervals (see Crozier, 1926). In the equation, μ usually represents heat of activation of reacting molecules, and is therefore expressed in calories. For biological processes this may be be of little significance because we know so little about the reactants involved, but since it may some day be possible to attach theoretical significance to μ we have quoted it in the units used by Crozier and others in biological work. In Fig. 6,

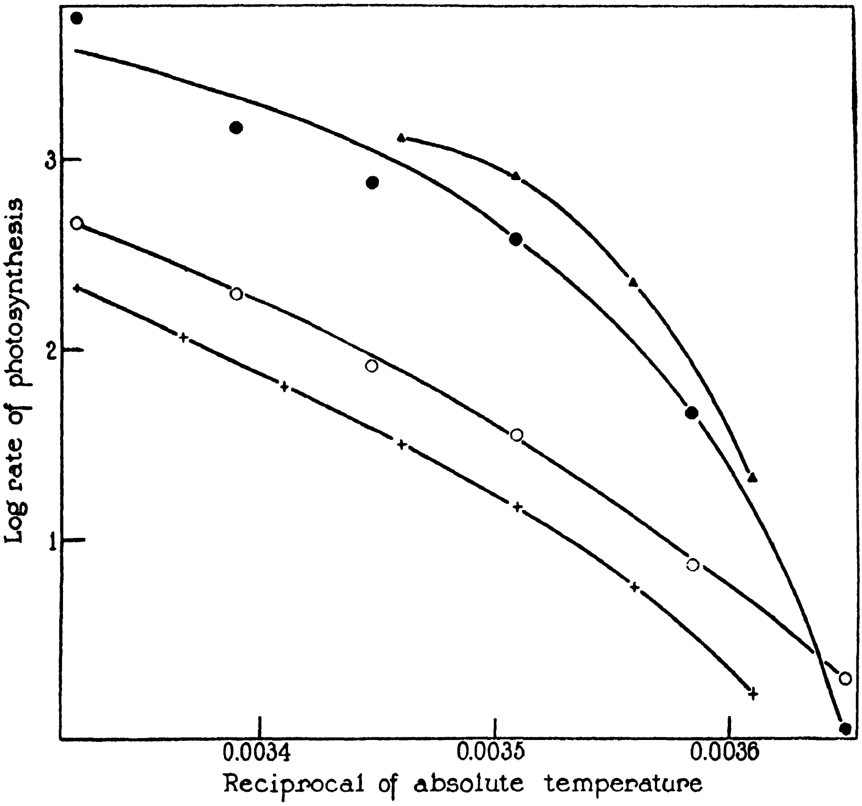

FIG. 6. Natural log of the rate of photosynthesis plotted against the reciprocal of the absolute temperature. Solid circles are for *C. vulgaris*, open for *C. pyrenoidosa*, triangles for *Gigartina*, and crosses for *Hormidium*. All measurements represent high intensity and carbon dioxide concentration.

where the natural log of the rate of photosynthesis is plotted against the reciprocal of the absolute temperature, μ represents the slope of the curves.

The solid and open circles are for *Chlorella vulgaris* and *C. pyrenoidosa* respectively. The triangles are for our measurements with *Gigartina*, and the crosses are calculated from van der Paauw's curve for *Hormidium* (1932, p. 558). The relative positions of the curves are arbitrary, and do not indicate relative rates of photosynthesis for the several organisms. Table V shows the rates of photosynthesis at the various temperatures, and the logarithms of the rates, adjusted to bring the curves to convenient relative positions in the figure. The maximum and minimum values of μ for each curve are also tabulated.

TABLE V

Temperature Characteristics of Photosynthesis for Various Algae. Data for Fig. 6

R is the rate of photosynthesis in arbitrary units. μ is the temperature characteristic or thermal increment defined by the equation

$$\log_e R_2 - \log_e R_1 = \frac{\mu}{2}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

where T is the absolute temperature. Only maximum and minimum values of μ are recorded.

Temperature	$\frac{1}{T_{\text{abs.}}} \times 10^6$	*Gigartina harveyana*		*Chlorella vulgaris*		*Chlorella pyrenoidosa*		*Hormidium flaccidum*	
		$\log_e R$	μ	$\log_e R$	μ	$\log_e R$	μ	$\log_e R$	μ
°C.									
1	3650			0.00	54000	0.27	18000		
4	3610	1.31	45000					0.19	23000
6	3584			1.66		0.86			
8	3559	2.35						0.75	
12	3509	2.92		2.58		1.55		1.17	
16	3460	3.12	6000					1.49	
17	3448			2.88		1.91			
20	3413							1.80	
22	3390			3.17		2.29			
24	3367							2.05	
28	3322			3.75	11000	2.66	12000	2.31	12000

The minimum values of μ are roughly the same for all four species of algae, but the *Gigartina* and *C. vulgaris* show a great increase in μ at the lower temperature, which is not shown by *Hormidium* and *C. pyrenoidosa.* If the data for *Gigartina* at low carbon dioxide concentration (open circles, Fig. 5) are plotted in this way, a curve nearly like those for *C. pyrenoidosa* and *Hormidium* is obtained. At present we can give no theoretical explanation for this, though it may be partly due to diffusion, as mentioned above. Crozier and Stier (1926) discuss other cases where the value of μ can be altered experimentally.

TABLE VI

Inhibition of Photosynthesis in Gigartina by Cyanide and by Phenyl Urethane

Cyanide experiment: 2.1 cm.^{2} algal material suspended in artificial sea water with carbonate mixture E. At the pH of this mixture the cyanide is practically all in the form of HCN at equilibrium.

Phenyl urethane experiment: 4.2 cm.^{2} algal material suspended in ordinary sea water saturated with 5 per cent CO_2 in air.

Temperature = 15°C.

Inhibitor	Concentration of inhibitor	Rate of photosynthesis	Inhibition	Rate after washing	Recovery
			per cent		*per cent*
KCN	0	33		28	
	$\frac{1}{20000}$ molar	17	49	26	86
Phenyl urethane	0	37			
	0.0075 per cent	14	62	41	100

We have found photosynthesis in *Gigartina* to be highly sensitive to traces of prussic acid and phenyl urethane. Removal of the urethane by washing results in complete reversal of the inhibition. In the case of cyanide, reversibility was not quite complete, even after short exposures to extremely low concentrations, and prolonged washing. Possibly the cyanide causes secondary injuries to the material. The inhibited photosynthesis in cyanide does not stay constant, but falls steadily, so our figures for percentage inhibition are arbitrary, being taken as soon as possible after introducing the cyanide. The results of our experiments on inhibition by cyanide and urethane are summarized in Table VI. Extreme sensitivity to cyanide and narcotics is a common characteristic of photosynthesis in green plants (*cf.* Spoehr, 1926, p. 171).

Two determinations of the photosynthetic quotient were made in artificial sea water saturated with 5 per cent carbon dioxide in air. Figures for these experiments are given in Table VII. The quotient obtained is close to unity, as might have been expected. Kniep's values for red algae also run close to unity, about −0.98. In neither case is the difference from unity significant, considering the accuracy of the methods used. Other determinations of the photosynthetic quotient of *Gigartina* were made in ordinary sea water saturated with 5 per cent carbon dioxide. These also gave values close to unity.

TABLE VII

Photosynthetic Quotient of Gigartina in Incandescent and in Mercury Light

The photosynthetic quotient, γ, is calculated by the formula

$$\gamma = \frac{K_{CO_2} \cdot k_{CO_2}}{K_{O_2} \cdot k_{O_2}} \cdot \frac{H \cdot K_{O_2} - h \cdot k_{O_2}}{h \cdot k_{CO_2} - H \cdot K_{CO_2}},$$

where the capital letters refer to the vessel with the larger fluid volume, the small letters to the other vessel. In each vessel, 4.2 cm.^{2} material suspended in artificial sea water without carbonates, saturated with 5 per cent CO_2 in air.

Incandescent light from a row of 60 watt lamps 8 cm. below vessels. Mercury light from an intense hot cathode mercury glow discharge tube close to vessels.

Temperature = 15°C.

Light	Volume of gas space	Volume of fluid including material	Vessel constants		Change of pressure	γ
	cc.	*cc.*			*mm.*	
Incandescent	$v_G = 9.93$ $V_G = 4.01$	$v_F = 3.3$ $V_F = 7.3$	$k_{O_2} = 0.95$ $K_{O_2} = 0.40$	$k_{CO_2} = 1\ 25$ $K_{CO_2} = 1.06$	$h = 12.4$ $H = 94.2$	−1.07
Mercury	$v_G = 10.18$ $V_G = 4.01$	$v_F = 3.3$ $V_F = 7.3$	$k_{O_2} = 0.97$ $K_{O_2} = 0.40$	$k_{CO_2} = 1.27$ $K_{CO_2} = 1.06$	$h = 9.5$ $H = 49.9$	−0.91
Average γ						−0.99

A few measurements of photosynthesis were made in different colors of light, in the hope of demonstrating whether or not phycoerythrin can act photosynthetically. The work was not completed because of lack of time, and we plan to make a more thorough investigation of the question later. For this reason we do not wish to give a detailed discussion at present. It may be worth while, however, to indicate what bearing our results have on the widely held opinion that red algae are enabled by their phycoerythrin content to utilize the shorter wave-lengths of light, and consequently to inhabit greater depths than other forms.

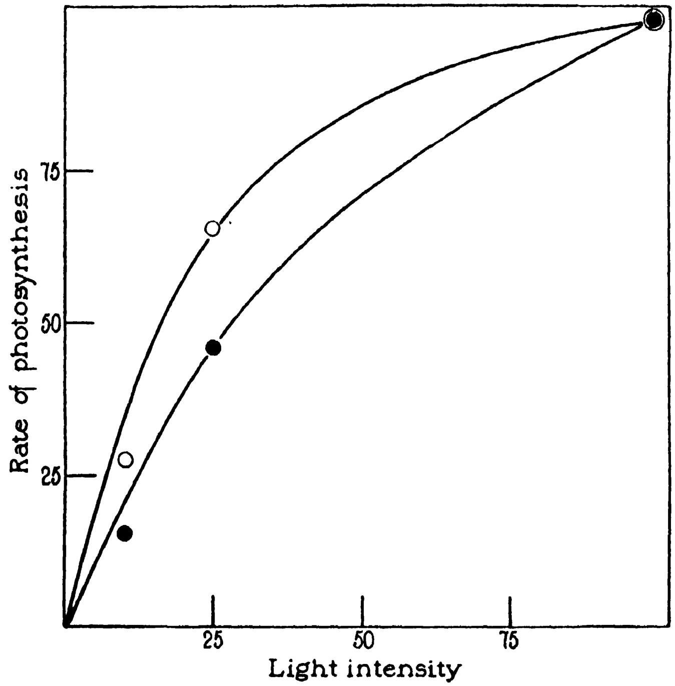

FIG. 7. Rough comparison of intensity curves for *Gigartina* and *Ulva*. Intensities and rates of photosynthesis are in arbitrary units. Open circles are for *Gigartina*, solid circles for *Ulva*. The highest rate of photosynthesis has been set at 100 in each case. Temperature = 15°C.

A number of investigators have compared the rates of photosynthesis by red and green algae in red and blue or green light, and found that the red algae do relatively better in the shorter wave-lengths than the green algae. We have been able to confirm this for *Gigartina* and *Ulva*. But von Richter (1912) has already pointed out that in certain cases at least, the results on changing from red to blue or green light can be duplicated by using a single color of light and changing from a higher to a lower intensity. Oltmanns (1892) reported that intensity was of greater importance than color, in obtaining satisfactory laboratory cultures of marine algae.

Von Richter's results suggest that photosynthesis in the red algae may generally be light-saturated at a lower intensity than in green algae. Fig. 7 shows that for *Gigartina* and *Ulva* this is certainly the case. Photosynthesis was measured at three intensities of white light for the two forms. The maximum photosynthesis in each case was set at 100. The *Ulva* responded to a fall of 75 per cent in light intensity by a fall of 55 per cent in photosynthesis, while the *Gigartina*, for the same fall in intensity, showed only a 33 per cent drop in photosynthesis.

TABLE VIII

Comparison of Rates of Photosynthesis in Gigartina and Other Organisms

Rates are expressed as mg. of CO_2 consumed in 5 hours per hundred square centimeters of material. The figure for *Gigartina* is from our data, and all other figures are from Kniep (1914).

Material	Temperature	Rate of photosynthesis
	°C.	
Gigartina Harveyana	16.0	68.0
Padina pavonia	22.7	13.74
Ulva lactuca	23.0	6.56
Porphyra laciniata	22.7	8.98
Helianthus annuus	20.7	109.0

Differences in the light intensity curves may therefore account for the results of Engelmann (1883), Harder (1923), Montfort (1930), and Ehrke (1932), which appear to have been erroneously interpreted as showing that the red algae could use green and blue light better than the green algae. This interpretation may be correct, but at present there seems to be no conclusive evidence for it.

We also submit a comparison of the absolute value of photosynthesis in *Gigartina* with figures for other marine forms, and for leaves of *Helianthus*, in Table VIII. Kniep (1914) has collected and tabulated a number of measurements, expressed in milligrams of carbon dioxide reduced per 100 cm^2. of material in 5 hours. Expressed in the same units, the rate of photosynthesis of a typical piece of our *Gigartina* compares favorably with the figure for *Helianthus*, especially considering the temperatures at which the two measurements were made. *Helianthus* is the most active of the land plants listed by Kniep. He represents the marine algae in general as having a much lower rate of photosynthesis than terrestrial plants. His finding supports our contention that the metabolism of marine algae has usually been studied under rather unfavorable conditions.

SUMMARY

A manometric method for measuring photosynthesis in marine algae is described.

Photosynthesis in the red alga *Gigartina harveyana* is shown to be similar in all important respects to photosynthesis in *Chlorella* and other Chlorophyceae.

We take this opportunity to express our thanks to members of the staff of the Hopkins Marine Station, especially Dr. Walter K. Fisher and Dr. C. B. van Niel, whose hospitality and cooperation made this work possible.

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SEARCH FOR MITOGENETIC RADIATION BY MEANS OF THE PHOTOELECTRIC METHOD*

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According to Gurwitsch's[1] theory, based upon investigations of the distribution of mitoses in growing organisms, an oscillatory phenomenon of unknown nature must be effective in producing mitoses. This leads to the further assumption that the dividing process itself is accompanied by the emission of such radiation and, further, part of this radiation must be emitted from the biological object. His fundamental experiment is said to prove this theory: the tips of onion roots are placed opposite each other at a small distance (approximately 1 mm.). One root serves as inductor, the other as detector. It was found that the number of mitoses in the side nearest to the inductor had increased in comparison to the side furthest from it.

Numerous experiments were undertaken by Gurwitsch and others[2] to prove the radiation character of this agent. Reflection and refraction experiments were devised to prove this. Absorption and spectroscopic experiments indicated that this radiation consisted of ultraviolet rays. While Gurwitsch and his school concluded from their experiments that the spectral region of the mitogenetic radiation lies between 1800 and 2500 A, Reiter and Gábor[2] found two mitogenetic maxima at 3400 and 2800 A, the presence of which is strongly denied by Gurwitsch.

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* A preliminary note on this subject was published in the *Pub. Health Rep., U. S. P. H. S.*, 1933, **48**, 1311.

[1] Gurwitsch, A., *Arch. mikr. Anat. u. Entwcklngsmechn.*, 1923, **100**, 11.

[2] Bibliographies are to be found in: Reiter, T., and Gábor, D., Zellteilung und Strahlung, *Sonderheft der wissensch. Veröffentlichungen aus dem Siemens-Konzern*, Berlin, Julius Springer, 1928, and Gurwitsch, A., Die mitogenetische Strahlung, Berlin, Julius Springer, 1932, 376.

The energy necessary for the production of such high frequency radiation is of chemical origin, as Gurwitsch assumes and tries to prove experimentally, and consists in reactions of the type of oxidation or proteolysis or glycolysis.

While the majority of the very numerous publications on mitogenetic radiation dealing with the biological side of the problem supports Gurwitsch's findings, a few[3] emphatically deny the effect and criticize his experimental methods. The problem has been attacked from the physical side also. Thus, two investigators, Rajewsky[4] and Frank and Rodionow[5] report physical proof for the existence of this radiation, while others, Schreiber and Friedrich[6] and Locher,[7] with similar experimental arrangements, could not detect any trace of it at all.

The use of yeast as biological detector for mitogenetic radiation is generally accepted at present and the increase in the number of budding cells in comparison with a control is said to give the order of magnitude of the effect. For physical detector, use is made of the photoelectric effect, either by employing the photographic plate or a device that works on the principle of the photoelectric cell.

It is the purpose of this paper to show that after careful exclusion of all possible sources of error the physical experiment does not give any proof for the existence of a mitogenetic radiation.

Theoretical Considerations

Assuming that mitogenetic radiation exists, it is possible, from theoretical considerations, to make a rough estimate of the minimum intensity of the mitogenetic radiation which can conceivably be detected. Having this intensity, it is possible to make an estimate of the sensitivity of the biological and physical methods used for the detection of the mitogenetic rays.

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[3] Taylor, G. W., and Harvey, E. N., *Biol. Bull. Marine Biol. Lab.*, 1931, **61,** 280. Richards, O. W., and Taylor, G. W., *Biol. Bull. Marine Biol. Lab.*, 1932, **61,** 113.

[4] Rajewsky, B., in Dessauer, F., Zehn Jahre Forschung auf dem physikalisch-medizinischen Grenzgebiet, Georg Thieme, Leipsic, 1931, 244.

[5] Frank, A. S., and Rodionow, G., *Naturwissenschaften*, 1931, **2,** 659.

[6] Schreiber, J., and Friedrich, W., *Biochem. Z.*, Berlin, 1930, **227,** 336.

[7] Locher, G. L., *Phys. Rev.*, 1932, **42,** 540.

As the intensity of the mitogenetic radiation is extremely small, it will simplify the matter to consider the emission and absorption of the mitogenetic radiation from the point of view of the quantum theory. According to this theory the emission and absorption of radiation is a discontinuous phenomenon and, further, there exists an "atom of radiation" called quantum, the magnitude of which is given by $h \times \nu$, where h is a constant and ν the frequency of the radiation as measured; *e.g.*, with a spectrometer in this way linking the wave theory of light with the quantum theory. Now, the emission from a mitogenetic inductor consists of ultraviolet light "quanta," emitted discontinuously; these quanta fall upon the biological or physical detector where they are absorbed. In the biological material, the absorption of a single quantum or of an integral number of quanta by a single cell is said to produce a mitosis; in the photographic plate a photochemical reaction will take place and in the photoelectric device the emission of a photoelectron will be the result of the absorption if, for the present, we do not consider the efficiency of these processes.

These circumstances make an estimate both of the intensity of the mitogenetic radiation and of the sensitivity of the methods possible. Beginning with the biological method: yeast is usually taken as detector. The diameter of a yeast cell is approximately 6 microns. In a yeast agar culture in which the cells lie packed closely together, we obtain approximately 30,000 cells per mm.^{2} for the top layer of cells (we need only to consider the top layer as ultraviolet radiation of a wave length of 1800 to 2500 A (this being the wave length of the mitogenetic radiation according to Gurwitsch) will not reach beyond this layer). The number of budding cells in yeast used as control is, according to Gurwitsch, about 10 per cent of the total number; *i.e.*, in this case 3000 per mm.^{2} Half an hour's exposure to a mitogenetic inductor placed in close proximity to the culture may give an increase in the number of budding cells of 50 per cent in the exposed area, as compared with the control. If we assume that each radiation quantum falling upon the culture is absorbed and furnishes the stimulus for the division of one cell, we have in our example 1500 quanta per mm.^{2} per 30 minutes or approximately 80 quanta per cm.^{2} per second as the intensity of the radiation coming from the inductor.

However, not all quanta falling upon the yeast will be absorbed; some will be reflected or scattered; not all of those absorbed will give the stimulus for a cell division since, according to their random distribution, some cells may absorb several quanta or a quantum may be absorbed by a cell already in the budding state. If an efficiency of 1/10 for this process (and this fraction is probably still too high) is assumed, as a lower limit for the intensity of the mitogenetic radiation an intensity of about 1000 quanta per cm.^{2} per second is obtained. Here the possible influence of secondary mitogenetic radiation within the irradiated medium has not been taken into account. This must be negligible, according to the data available for yeast agar cultures.[8] Frank and Rodionow[9] estimate the intensity of the mitogenetic radiation to be from 100 to 1000 quanta per cm.^{2} per second.

If this value of 1000 quanta per cm.^{2} per second is taken as the probable intensity of the mitogenetic radiation, this radiation, according to the above example, will produce in 1 mm.^{2} of a closely packed yeast culture (30,000 cells with 3000 budding cells) an increase of 1500 budding cells within 30 minutes. So great a number of cells cannot be counted in a single experiment. Perhaps 1/10 of this number can be counted. This would mean, out of 3000 counted cells of the example, 300 budding cells in the control and 150 additional budding cells in the irradiated sample would be counted. It is doubtful whether such a finding has any meaning at all, as long as little is known about the natural fluctuations of budding cells within a yeast culture. A positive result of a mitogenetic experiment would be obtained only if the number of budding cells in the experiment were to exceed the number of budding cells in the control by at least three times the mean error obtained by a series of counts of budding cells in a normal yeast culture. Due to the limitations of the subjective method of counting, one cannot increase the sensitivity of the biological method by counting a larger number of cells. This could be done only by the use of an objective method. Gurwitsch[10] describes two such methods, a nephelometric and mycetocritic one. The data given, however, are insufficient to permit an estimate as to sensitivity and errors.

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[8] Potozky, A., *Biol. Zentr.*, 1930, **50**, 712.

[9] Gurwitsch, A., Die mitogenetische Strahlung, Berlin, Julius Springer, 1932, 47.

[10] Gurwitsch, A., Die mitogenetische Strahlung, Berlin, Julius Springer, 1932, 16–18.

In order to make a comparison between the biological and physical methods as to sensitivity, let us assume that the above example gives a reliable positive result for an intensity of mitogenetic radiation of 1000 quanta per cm.^{2} per second acting during 30 minutes. This interval was arbitrarily chosen as being sufficient to register any positive effect and yet insure the activity of the specimen during the period of observation.

We proceed now to a discussion of the physical methods and their sensitivity.

The simpler of the two methods is the one employing the photographic plate. A just perceptible blackening of a sensitive photographic emulsion is produced by a light energy of 2×10^8 quanta per cm.^{2}.[11] As the intensity of the mitogenetic radiation was assumed to be 1000 quanta per cm.^{2} per second, 2×10^8 quanta would be obtained in a time of irradiation of 2×10^5 seconds = 55 hours. A time of exposure of a photographic plate to mitogenetic radiation of 100 hours should produce an easily perceptible blackening of a photographic plate.

The sensitivity of a photoelectric cell arrangement can be determined as follows. A cell of medium sensitivity will have an efficiency[12] of approximately 1/1000 for the wave length at its maximum sensitivity; *i.e.*, for 1000 impinging light quanta, 1 photoelectron will be liberated. With a photoelectric cell of the customary type connected into circuit with a battery and an electrometer or galvanometer, the measurement of currents of the order of magnitude of a few electrons per second is extremely difficult. Such a measurement, however, is made comparatively simple by combining the principle of the photoelectric cell with a so called Geiger counter tube. Such a counter tube consists of a fine wire axial in a metallic cylinder under a gas pressure of about 5 cm. of Hg. By applying a negative potential of approximately 1500 volts to a metallic cylinder and grounding the wire over a high resistance of the order of magnitude of 10^9 ohms, an electron, liberated from the walls of the tube by any kind of radiation, will travel toward the wire, producing on its path an ionic cloud by impact, resulting in a relatively strong current impulse through the high resistance to ground. This current impulse can be recorded by a string electrometer or by a suitable amplifier with mechanical recorder, as will be shown later. To make such a counter sensitive to light, the walls of the tube must be made of a photoelectric metal and a window provided for the impinging light. This method was first used in testing for mitogenetic radiation by Rajewsky[4]; the other authors previously mentioned used arrangements of a similar kind.

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[11] Geiger, H., Handbuch der Physik, Berlin, Julius Springer, 1926, **23**, 628.

[12] Wien, W., and Harms, F., Handbuch der Experimentalphysik, Leipsic, Akademische Verlagsgesellschaft, 1928, **23** (2), 1205, Table 17.

Taking the photoelectric efficiency as 1/1000 and assuming a 30 minute exposure to a radiation intensity of 1000 quanta per $cm.^2$ per second we obtain for a window of 1 $cm.^2$ a liberation of 1800 photoelectrons in such a tube.

Although the efficiency of the photoelectric process is much smaller than that of the biological process in the detector, the sensitivity of the photoelectric method is much higher, due to the fact that in the biological counting method use can be made of an irradiated area of a fraction of a millimeter only, while in the physical method detecting areas of 1 $cm.^2$ or even more can be employed. As will be later described, counter tubes with a window area of 6 to 7 $cm.^2$ were used which would increase the number of photoelectrons of the above calculations by a factor of 6 or 7.

To sum up: For an assumed intensity of the mitogenetic radiation of 1000 quanta per $cm.^2$ per second,—a probable value for the intensity established by the experimental data given above—the biological method, consisting in counting yeast cells, produces a perceptible effect in the detector, the photographic method should give a perceptible blackening of a photographic plate, and the photoelectric method should yield an effect far above the sensitivity threshold of that method.

EXPERIMENTS

After considering and discussing the different methods, the physical experiments—photographic as well as photoelectric—which were undertaken to study the problem of the existence of mitogenetic radiation will now be described.

The photographic experiments were carried out in the following way: Three light-tight boxes 22 × 15 × 22 cm. were prepared with a sliding lid in front, to the bottom of which metal plate holders were fastened. The metal lid of these plate holders had an opening of 4 × 5 cm. This opening was lined with velvet. Quartz plates 6 × 8 cm. in size, 0.5 mm. thick, selected as to transparency, were placed upon the velvet, thus effectively sealing the photographic plate against any possible chemical influence by volatile substances from the onions or onion-base pulp used in the experiments. To the center of the quartz plate a cylinder of pyrex glass was sealed with de Khotinsky cement; the cylinder was 25 mm. in diameter and 2 cm. high in the experiments with onion pulp and 5 cm. high in the experiments with onion roots. The transparency of the quartz was tested spectroscopically down to a wave length of 1800 A. The loss of intensity for this wave length was not higher than 20 per cent. As the distance of the onion-base pulp or onion roots from the photographic emulsion was not greater than 1 to 2 mm., practically all radiation emitted by the biological material into the lower hemisphere was necessarily absorbed by the photographic emulsion. As photographic material, Eastman Speedway plates were chosen; in some of the experiments these plates were sensitized by a thin coating of mineral oil (Nujol) to overcome the possible objection that the sensitivity of the photographic plate decreases considerably for short ultraviolet on account of absorption by the gelatine of the emulsion. Onion-base pulp was prepared from selected ordinary onions sprouting vigorously. The pulp was changed every 2 hours. In the case of the experiments with onion roots, vigorously sprouting onions were chosen with sprouts of a few centimeters in length and placed on top of the glass cylinder partly filled with a 0.1 per cent solution of KCl. Care was taken that a great number of root tips (about 10 to 20) touched the quartz plate. These onions were inspected every day and replaced every 2nd day. All operations of changing onion-base pulp or onions were carried out in complete darkness; a special device was provided insuring that the biological material was always set at the same place.

Every box was provided with an opening on top and bottom, carrying a rubber hose through which air was gently sucked by means of an aspirator.

All plates were developed in a hydrochinone solution of twice the normal strength; the sensitizing oil being removed in an ether bath before the developing process. Hydrochinone was chosen as it gives very strong contrasts.

Table I gives the experimental data of the photographic experiments.

All these plates showed a rather strong fogging which, however, was identical with that of a control plate taken from the package and developed with the same developer for 4 minutes. This fogging was due to the strong concentration of the developer used. In spite of the fog, any slight difference in density could have been detected. Not the slightest difference in density could be found in any of the six plates which could be attributed to an effect from radiation coming from the onion pulp or roots.

There are three possible objections to the photographic method. The first one is the uncertainty as to whether all the biological material used in the experiments with onion-base pulp was equally active and whether it remained active during the total time of 2 hours for which time every single preparation was used. However, there is little doubt that the onion tips used for Experiments 2 and 4 were active.

TABLE I

No.	Biological material	Total time of exposure	Photographic material	Time of development	Remarks
		hrs.		*min.*	
1	Onion-base pulp	106	Eastman Speedway sensitized	7	Onions grown in dark
2	Tips of onion roots	120	" "	4	Onions grown in daylight
3	Onion-base pulp	106	" "	7	" "
4	Tips of onion roots	168	" "	8	" "
5*	Onion-base pulp	184	Eastman Speedway unsensitized	3–4	" "
6*	" "	184	" "	3–4	Onions grown in dark

* I am very much indebted to Dr. C. H. Binford for carrying out these two experiments.

The second possible objection is that the intensity of the mitogenetic radiation in reality is weaker than the estimate previously given. That this is improbable has already been pointed out. However, if it were actually only $\frac{1}{3}$ of the assumed value, it should nevertheless have been detected in the case of the onion root experiments by the photographic method.

Finally, the exponent in Schwarzschild's law ($s = i \times t^p$, where s = density, i = intensity, and t = time of exposure) which lies between 0.9 and 1.1 for different photographic emulsions may have been much smaller than 1 for the photographic emulsion used in these experiments. There is nothing known about the value of this exponent for short ultraviolet; however, it is probable because of several considerations that it differs but little from 1. Assuming a value of 0.9 for the exponent, the effective time of an experiment lasting say 160 hours would be reduced to $160^{0.9} = 96$ hours, an interval which still should be sufficient to produce a perceptible blackening.

Because of negative results, further photographic experiments were abandoned, especially as the photoelectric method permits an experimental arrangement the sensitivity of which is such that it can detect radiation intensities far below the estimated intensity of the mitogenetic radiation. Although by themselves the results of the photographic experiments are not conclusive, they serve to corroborate the more clear-cut results obtained with the photoelectric method.

The principle of the photoelectric counter tube has already been explained. After testing different kinds of counter tubes, a tube consisting entirely of quartz was finally adopted. For counter tubes used in cosmic ray work, for instance, any metal tube with ends sealed by means of rubber stoppers and cement will do, as the sensitivity of the tube is independent of the surface properties of the metal and of the filling gas. However, in tubes to be used either for ultraviolet or visible radiation work, great care has to be taken that the surface conditions of the photoelectric metal remain unaltered, as time goes on, by chemical changes such as oxidation. Otherwise, considerable changes in sensitivity will occur. For this reason, the counter tubes used in this work consisted of thin walled quartz tubes (wall thickness approximately 1 mm., length 10 cm., diameter 2 cm.) of high transparency for ultraviolet light. The transparency of the tubes was tested with a quartz spectrograph and it was found that absorption in the quartz was negligible down to 2200 A, the limit of the spectrograph. An area of 6 to 7 cm.^2 was flattened out to serve as window. The wire of the tube consisted of tantalum, 0.02 cm. diameter, and was connected to two thick copper wires held in place by 2 quartz capillaries at the end of the tubes. These copper leads were sealed vacuum tight into the capillaries by silver chloride cement. Three side tubes were provided, one for exhausting purposes, one for distilling in the metal, and a third for carrying a wire cemented in by silver chloride and making contact with the photoelectric layer. The tube was exhausted by a mercury diffusion pump with liquid air trap for 8 to 10 hours, heated several times with a blow torch to yellow heat; the wire was degassed for 3 hours by heating it with a battery. Spectroscopically pure cadmium was then distilled into the tube, the wire and window were heated to remove any cadmium deposit, and pure argon was filled in to a pressure of 4 to 6 cm. of Hg. Counter tubes prepared this way do not show any change in sensitivity with time.

Although cadmium is not a very sensitive photoelectric metal, it was nevertheless chosen, because its sensitivity increases rapidly for wave lengths shorter than

3100 A, where, according to Gurwitsch, the mitogenetic spectrum lies. A further advantage is that visible stray light of longer wave lengths will not affect the counter tube, since the threshold sensitivity of a cadmium counter tube was found to lie between 3400 and 3500 A. Moreover, shielding for very small amounts of stray visible light would have been very difficult inasmuch as the work could not be carried out in complete darkness. Counter tubes with zinc as photoelectric metal were also made. They were similar to the cadmium tubes, both in sensitivity and threshold wave length. The counter tubes were connected into circuit both with an amplifier operating a mechanical counter, and with a string electrometer provided with a photographic recorder. Fig. 1 gives the experimental arrangements.

The negative pole of a dry cell battery[13] of 1500 volts with means for changing the tube potential in steps of 1.5 volts and a voltmeter consisting of a microammeter in series with twenty resistors of 10^6 ohms each was connected to the cadmium deposit. The axial wire grounded over a resistance of 2×10^9 ohms[14] was connected to the fiber of a string electrometer in which the potential difference of the plates was 100 volts. In this connection it may be stated that the deflection of the string is approximately proportional to the voltage. Coupling to the amplifier was effected by means of a variable condenser. The amplifier had two resistance-condenser coupled stages. The tube of the last stage was a thyratron, the plate current of which, limited by means of a resistance to 80 milliamperes, was large enough to operate a mechanical counter of the type used for counting telephone messages. The movable arm of this counter carried a small pin, breaking a contact switch in the thyratron circuit at the highest position of the arm. This arrangement is necessary since the grid of the thyratron becomes ineffective the moment it has "triggered" the gaseous discharge between cathode and anode.

The photographic recording device used for recording the movements of the string of the electrometer and studying the character of the discharge consisted of an electric motor driving a photographic recording paper through a train of gears having a variable ratio so as to obtain different recording speeds. Most of the experiments with biological material as a radiator were counted with the mechanical counter as well as photographically recorded. The number of counts obtained by the two methods were identical within a few tenths of 1 per cent.

Since the counter tubes just described are sensitive not only to ultraviolet radiation but also to radiation coming from radioactive substances in the ground, air, and walls of the building, as well as to cosmic radiation, every counter tube will give a residual effect; *i.e.*, the apparatus will record a certain number of counts per minute due to the electrons liberated by these radiations, the number of which depends, other things being equal, upon the cross-sectional area of the tube and its sensitivity. In every experiment with another source of radiation, this back-

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[13] Burgess P. L. batteries.

[14] Manufactured by the S. S. White Dental Co., New York.

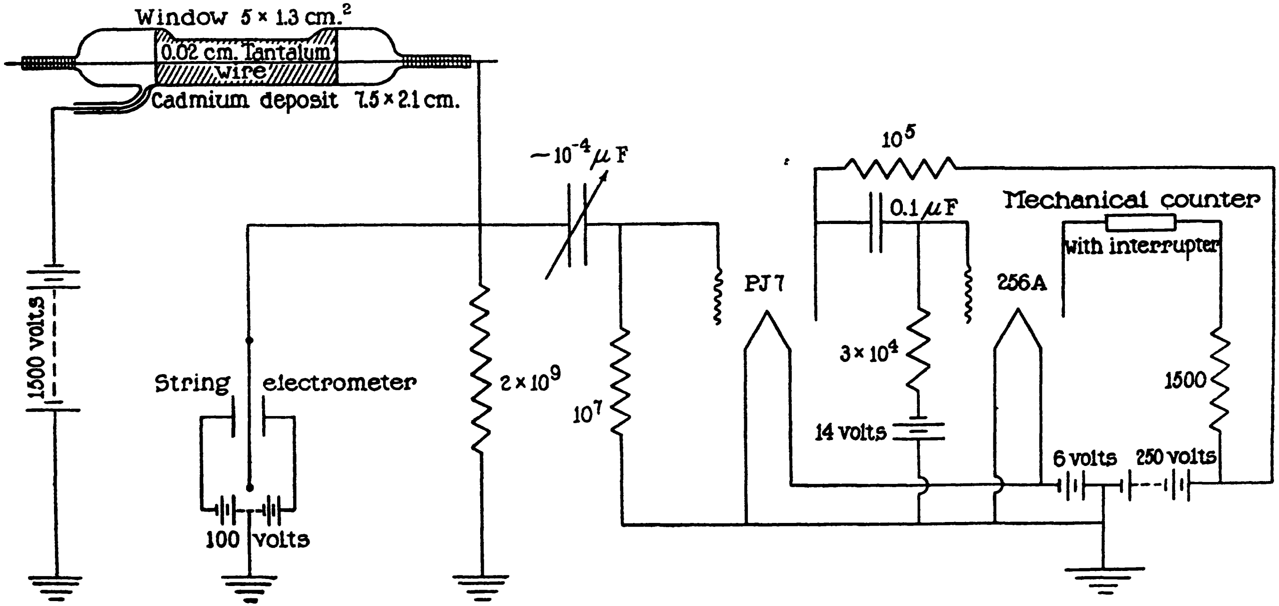
Window 5 × 1.3 cm.^{2}
0.02 cm. Tantalum wire
Cadmium deposit 7.5 × 2.1 cm.
1500 volts
String electrometer
100 volts
2 × 10^9
~10^{-4} μF
10^7
PJ7
10^5
0.1 μF
3 × 10^4
14 volts
256A
Mechanical counter
with interrupter
1500
6 volts
250 volts

Fig. 1

ground radiation has to be taken into account and its relative intensity can only be found from the difference between the number of counts produced by the source plus the background radiation and the number of counts of the background radiation within the same interval of time. As the time during which a biological object acting as a radiator remains alive is relatively short, it can readily be seen that a strong background radiation can mask the effect of a weak additional radiation. On account of its random distribution it renders the number of counts produced by it in a given time subject to the statistical error, the magnitude of which is given by the square root of the total number of counts. Therefore, the trustworthiness of a measurement of the intensity of an additional weak radiation depends upon the magnitude of this additional effect. This will be discussed later. Consequently, it is necessary to cut down as much as possible the effect of the background radiation without, at the same time, impairing the sensitivity of the counter tube. For this reason the counter tube was enclosed in a lead box with walls of sufficient thickness to surround this tube on all sides with 10 cm. of lead. This lead was, of course, selected as to its freedom from radioactive substances. Although a lead shield 10 cm. thick cuts out only the softer components of the background radiation, nevertheless the shield effected a reduction of approximately 50 per cent in the number of counts from this source.

Experiments with biological material as a possible source of radiation were carried out for the most part as follows: First the effect of the background radiation was measured by counting the number of counts during a certain time, usually 30 minutes. Then the biological object was placed upon the window; usually a few drops of tap water or distilled water or potassium nitrate solution or an inorganic serum solution[15] were added to prevent drying out as an air current had to be passed through the lead box to prevent the formation of a water film on the quartz of the counter tube which would have acted as a short circuit to ground. Several tests were made of the biological material used in these experiments as to viability before and after the 30 minutes of the experiments. In all the tests the tissue was viable after the experiments.[16] Finally the test for the background radiation was repeated after removing the biological material.

The number of counts produced by the counter tubes was, on an average, approximately 20 per minute; *i.e.*, in half an hour about 600 counts were recorded. As already stated, this number is subject to a statistical error $= \sqrt{600} = \pm 24.5$ counts. The number of counts obtained from another source of radiation added to the number of counts of the background radiation is likewise subject to a statistical error. To obtain an estimate of the weakest possible intensity of a source of radiation that we still can measure without involving a statistical error large enough to invalidate the result, we shall arbitrarily assume that in the presence of a radiator, an increase in the number of counts which is twice the statistical error of the number of counts produced by the background radiation in the same time is an indication of the presence of additional radiation and we shall call this the "minimum effect." Even then only a series of observations, all of which show an effect of the same order of magnitude, will furnish definite proof of the existence of an additional effect. 600 counts per 30 minutes were, on an average, observed as the effect of the background radiation; twice the statistical error is 49 counts. The minimum effect would be observed if the background radiation and the additional radiation together produce 649 counts per 30 minutes. The statistical error of the difference of 49 counts would be $\sqrt{600 + 649} = 35.6$ counts = ±72 per cent.

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[15] Shear, M. J., and Fogg, L. C., *Pub. Health Rep., U. S. P. H. S.*, 1934, **49**, 229.

[16] I am very much indebted to Dr. L. C. Fogg who carried out these tests.

These considerations show the importance of cutting down the background radiation and of extending the time of duration of an experiment as both factors will increase the sensitivity of the arrangement.

From the minimum effect of approximately 50 additional counts in 30 minutes; it is possible to calculate the theoretical number of light quanta which one should be able to detect. 50 counts in 30 minutes correspond to 0.03 counts per second. The photoelectric efficiency being of the order of magnitude of 1:1000, 0.03 counts per second will be produced by 30 quanta per second that have passed through the window. The area of the window being approximately 6 cm.², a theoretical number of 5 light quanta per cm.² per second is obtained, which should be detected in a series of experiments. This is far below the theoretical minimum intensity of the mitogenetic radiation.

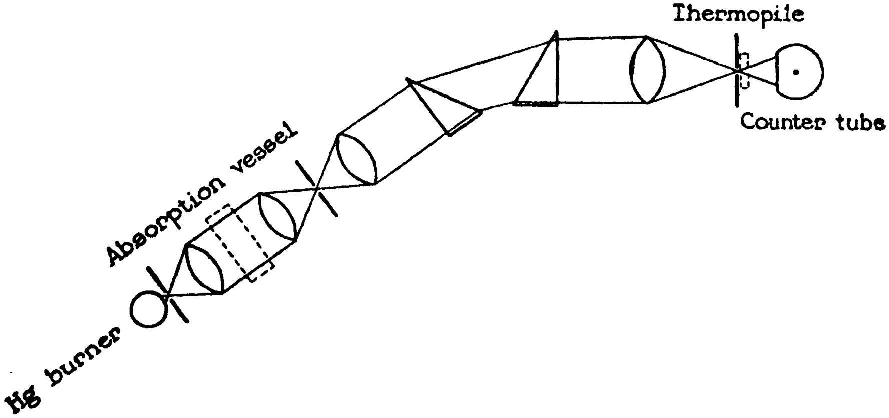

FIG. 2

The experimental calibration of the counter tubes was carried out with an arrangement shown in Fig. 2. The monochromator used was manufactured by Bausch & Lomb; according to the manufacturer, the amount of stray radiation reaching the exit slit being of the order of magnitude of a few tenths of 1 per cent for a slit width of 0.05 mm. As sources of light a D.C. mercury arc lamp, an A.C. mercury arc lamp, and an A.C. cadmium arc lamp were used. The final measurements were carried out with the D.C. mercury arc lamp. A small slit of 2 × 8 mm. was placed directly in front of the arc lamp to obtain as source of light an area of the same luminous intensity per unit area. An image of this slit was thrown upon the entrance slit of the monochromator by the two condenser lenses. A vessel with an absorbing material of known density could be placed between these two lenses to decrease the intensity. Directly behind the exit slit a vacuum Coblentz thermopile was placed, mounted in a carrier which could be moved up and down so that the thermopile or the counter tube could alternately be exposed. The diverging beam emerging from the exit slit was of almost uniform intensity and well defined cross-section. The counter tube was placed at such distance that the cross-section of the beam corresponded to the dimensions of the window of the tube.

The calibration of the counter tube in quanta per $cm.^2$ per second was carried out in the following way. The intensity of the Hg line 2536 A was measured with the thermopile which was calibrated in absolute units against a standard lamp. Then the intensity of the line was decreased to $1:9 \times 10^9$ of its value by putting between the two condenser lenses or between exit slit and counter tube an absorption vessel containing a solution of $K_2Cr_2O_7$ of known concentration. The extinction coefficient of $K_2Cr_2O_7$ was carefully determined with the thermopile by using a series of more dilute solutions of known concentration. In addition, checks of the validity of Beer's law were made, although it could be assumed that Beer's law was valid for the concentration of 8 gm. in 10 liters of distilled water used to produce the reduction in intensity given above ($1:9 \times 10^9$). The law was found to be valid within the experimental error. After removing the thermopile, this weak radiation fell upon the counter tube. As the intensity of this beam is known in quanta per $cm.^2$ per second, the number of counts (difference of background counts and background plus radiation counts) now given by the counter tube in a certain time corresponds to the number of quanta passing through the window in this time. From this value the minimum effect could be calculated. For the Hg line 2536 A, 50 additional counts in 30 minutes are produced by an intensity of 10 to 15 quanta per $cm.^2$ per second falling upon the window of the counter tube. As the steeply rising branch of the sensitivity-wave length curve for cadmium extends to still much shorter wave lengths up to the wave length at which the absorption in quartz begins to become considerable, it is obvious that the counter tubes will approach the calculated minimum effect in the region of the wave length of the mitogenetic radiation. For cadmium, the measured sensitivity for the wave length 2300 A is 1.7 times that for the wave length 2536 A.[17] For these measurements as well as for the biological measurements to be reported later, the voltage on the counter was raised as high as possible, to obtain highest sensitivity; *i.e.*, near to the point at which erratic operation of the counter tube begins, due not to the incident radiation but to spontaneous discharges within the tube. However, at the applied voltage the counter tubes work normally for any length of time if certain precautions are taken.

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[17] International Critical Tables, New York, McGraw-Hill Book Co., Inc., 1929, **6**, 68, Table 3.

The electrostatic field within the counter tubes, due to the arrangement of a thin wire in the axis of a conducting cylinder, is logarithmic, which means that almost the entire potential difference between cylindrical electrode and wire lies within a narrow region around the axial wire. In this region the ionic cloud is produced by the photoelectron released from the wall, and the consequently formed secondary electrons which charge the wire and produce the impulse which is recorded. The high resistance of 10^9 ohms prevents the immediate removal of this charge, resulting in diminishing the potential difference of the electrodes to a value for which the potential difference is insufficient to produce additional ions by impact. Thus the discharge stops; the wire discharges, which brings the potential difference between the electrodes once more to its original value. The counter tube is then ready for the next discharge. It is evident that the window will have an influence on this logarithmic field, especially as it consists of quartz, the insulating properties of which are very high. During the operation of a counter tube provided with a window, stray ions will go to the window and produce a change in the field around the wire, thus influencing the sensitivity (counting rate) of the tube until an equilibrium is reached. The greater the sensitivity of the counter tube, the more noticeable is this effect.

During the first series of experiments in which the counter tube was used in the study of mitogenetic radiation, somewhat erratic results were obtained which, nevertheless, seemed to point toward the existence of this radiation. In the endeavor to exclude any spurious effect, experiments were made with substances which could not possibly emit any radiation, *e.g.* water, and effects consisting in an increased counting rate were obtained. It was finally found that mere touching of the window produced an effect. After what has been said about the influence of the window upon the operation of the counter, it seemed obvious that all the effects just noted were produced by disturbing the field in the counter tube. When counting rates were taken from minute to minute, it was found that the effect gradually died off until the equilibrium was again reached. In the case of biological material this might be falsely interpreted as due to gradual loss in viability.

A series of experiments was undertaken to show the magnitude of this spurious effect.

Table II gives data on some of these experiments.

Especially interesting are the experiments with tubes of glass and quartz, respectively, closed at one end and partly filled with water. Since these tubes were in contact with only a small part of the window, one would expect a comparatively small effect. Both tubes were cleaned before placing them on the window by rubbing them gently with cheese-cloth. Although both tubes were of the same size, the quartz tube produced a much larger effect while that of the glass tube is somewhat larger than the statistical error. Upon wiping off the tubes with moist tissue paper, in the case of the quartz tube the effect was decreased, while in the case of the glass tube it disappeared. This shows that the larger effect with the quartz tube was due to charges on the quartz. Due to the high insulating power of quartz, an electric charge is easily obtained and can be removed only with difficulty. This is true for glass also, but the charge is much smaller in this case, as the insulating properties of glass are inferior to those of quartz. It can be shown by means of an electroscope that quartz, once well rubbed, retains its charge for several hours when kept in a dry room, while that of glass will disappear in a few minutes. Since, in experiments for demonstrating the presence of mitogenetic radiation by putting the biological material in both glass and quartz tubes in order to show its ultraviolet nature, tubes will be rubbed clean to avoid possible absorption, the effect shown above gives a possible explanation of the positive results that have been reported in such experiments.

TABLE II

Counting rate per min. for uncovered window	Material put on window	Counting rate per min. for covered window
15.6 ±0.9	Piece of lead foil, not grounded	20.9 ±1.4
25.6 ±1.6	Piece of lead foil, grounded	47.8 ±2.2
21.5 ±1.0	Water, not grounded	28.2 ±1.7
24.7 ±1.0	Quartz tube slightly rubbed	38.5 ±2.0
23.0 ±1.1	Glass tube slightly rubbed	29.0 ±1.7
23.5 ±1.5	Glass tube wiped off with moist paper	26.6 ±1.6
22.9 ±1.5	Quartz tube wiped off with moist paper	32.6 ±1.8

The experiments with metal foil or water on the window may explain why it is that some of the investigators who employed photoelectric methods for the demonstration of mitogenetic radiation found this radiation to be present while others did not. In Schreiber and

Friedrich's, as well as in Locher's experiments, the biological material was not placed directly on the window; there was an air space between. Rajewsky, on the other hand, placed his material on the window. Frank and Rodionow, so far as can be learned from their brief publication, apparently tetanized a frog's muscle by means of an induction coil in front of their window, thus creating violent electric disturbances which must have influenced the static field of their cell.

The data of an experiment with onion roots may be given to show how large this spurious effect was in some cases:

September 4, 1931. Onion Root Experiment

Counter voltage: 1600 volts.

1. Background radiation	903 counts in 40′	22.6/min. ±0.7
2. Onion roots on window	2187 counts in 50′	43.7/min. ±0.9
3. Chloral hydrate (1 per cent) dropped on onion roots	2962 counts in 50′	59.2/min. ±1.1
4. Background radiation	904 counts in 40′	22.6/min. ±0.7

These effects disappeared after proper shielding of the window was effected.

The shielding consisted in surrounding the tube with a grounded metallic wall, with an opening for the window. In order to keep the window at the same potential during the measurement of the background radiation as well as during measurements with biological material, the window was covered with any one of the several liquids previously mentioned, which were used in some cases to keep the biological material moist, and a wire connected the liquid to ground. Inasmuch as tap water or the other liquids are conducting to some degree the surface of the window was kept continuously at the same; *i.e.*, ground potential. For the experiments with biological material the water was removed and enough material put on the window to cover it completely.

Another means of preventing spurious effects would be the placing of the biological material at some distance from the window, say 1 to 2 cm. But this would necessarily result in loss of intensity which, if possible, has to be avoided.

With this arrangement numerous tests for the presence of mitogenetic radiation were made.

The biological material tested consisted mainly of onion-base pulp and tips of onion roots. Mouse sarcoma 180, mouse embryo tissue, and tetanized frog muscle, all alleged to be excellent radiators, were likewise tested. The results of some of the experiments are given in Table III.

TABLE III

Results of Some of the Tests

Biological material	Time	No. of counts		
		Control	With biological object	Control
	min.			
Onion-base pulp	30	529 ±23.0	523 ±23.0	534 ±23.0
" " "	30	562 ±23.6	562 ±23.6	516 ±22.7
" " "	30	552 ±23.5	534 ±23.0	497 ±22.3
Mouse embryo	30	543 ±23.3	552 ±23.5	515 ±22.7
" "	30	646 ±25.4	611 ±24.7	617 ±24.8
Onion-base pulp	30	535 ±23.1	521 ±22.8	527 ±22.9
Mouse embryo	30	507 ±22.5	517 ±22.7	543 ±23.3
Onion root	40	643 ±25.3	643 ±25.3	641 ±25.3
" "	40	981 ±31.3	977 ±31.2	898 ±29.9
" "	40	1112 ±33.3	1092 ±33.0	1159 ±34.0
Frog muscle (tetanized)	30	656 ±25.6	673 ±25.9	669 ±25.8
Mouse sarcoma	40	504 ±22.4	521 ±22.8	624 ±24.9
" "	40	597 ±24.4	582 ±24.1	625 ±25.0

DISCUSSION

The data given show that no mitogenetic radiation could be detected. If mitogenetic radiation exists at all, its intensity must be smaller than the minimum effect as established for the counter tube; *i.e.*, its intensity must be smaller than 10 to 15 quanta per cm.^2 per second. The estimate of intensity, as given at the beginning, gives as the smallest—though highly improbable—value approximately 100 quanta per cm.^2 per second. The counter tube would have detected such an intensity. Therefore we must conclude that there is no physical proof for the existence of mitogenetic radiation.

Consideration of the energy content of the chemical reactions which, according to Gurwitsch,[18] are responsible for the emission of the mitogenetic radiation and which may be reactions of either oxidation or proteolytic or glycolytic character shows that their heat of reaction is insufficient for the production of ultraviolet radiation of a wave length between 1800 and 2500 A. The energy necessary to produce quanta of a wave length of 2000 A is 142.2 kg. cal. So far as known there is no biological reaction which will produce in a single step a heat of reaction greater than approximately 70 kg. cal. It must either be assumed that there exist unknown reactions of the above type which produce sufficient energy, but in this case the atoms or radicals would have to take part in the reaction, or that in the case of reactions with insufficient energy, rare single processes may occur which result in the production of an ultraviolet quantum corresponding to the wave length of mitogenetic radiation. There is no physical or physicochemical evidence that either of these cases is possible.

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[18] Gurwitsch, A., Die mitogenetische Strahlung, Berlin, Julius Springer, 1932, 47–68.

SUMMARY

The intensity of mitogenetic radiation was estimated from data given by Gurwitsch.

The sensitivity of the biological method and of the physical methods were compared.

With onion-base pulp and onion roots as mitogenetic inductors, the photographic method gave no perceptible blackening for exposures up to 184 hours.

A photoelectric counter tube was described with cadmium as photoelectric metal. Its sensitivity was such that a radiation intensity of 10 to 15 quanta per cm.^{2} per second of the Hg line 2536 A was detectable.

Spurious effects produced by the counter tube were described and means for their avoidance given.

A number of different biological materials, all supposed to be excellent mitogenetic radiators, were investigated by means of the counter tube. No mitogenetic radiation could be detected.

Addendum.—After the manuscript was written, a paper was published by Gray, J., and Ouellet, C., *Proc. Roy. Soc. London, Series B*, 1933, **114**, 1, describing experiments on mitogenetic radiation with a photoelectric Geiger counter tube of a sensitivity of 50 quanta per cm.^{2} per second at 2500 A which gave no indication of a radiation from fertilized eggs of sea urchins, cultures of active spermatozoa, or of growing yeast. Spurious effects due to condensation of water vapor or other volatile substances on the counter tube were observed and means for their prevention given.

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